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(54) Title: **β -CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER**

(57) Abstract: A recombinant adenovirus (Ad-Mini-ME) which constitutively expresses the central third of APC includes all of the known β -catenin binding repeats. When expressed in colon cancer cells, Ad-Mini-ME blocked the nuclear translocation of β -catenin and inhibited β -catenin/Tcf-4-mediated transactivation. Accordingly, expression of endogenous targets of the APC/ β -catenin/Tcf-4 pathway were down-regulated. Ad-Mini-ME infection of colorectal cancer cell lines with mutant APC but wild-type β -catenin resulted in substantial growth arrest followed by apoptosis. These findings suggest that the β -catenin binding domain in the central third of APC is sufficient for its tumor suppression activity.



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β -CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant CA57345 awarded by the National Institutes of Health.

This application is a continuation-in-part of application Serial No. 09/003,687 filed January 6, 1998, which is a division of application 08/821,355 filed March 20, 1997, now U.S. Patent No. 5,851,775.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of cancer diagnostics and therapeutics. More particularly it relates to methods for diagnosing and treating cancers associated with APC or β -catenin mutations.

BACKGROUND OF THE INVENTION

Mutations of the adenomatous polyposis coli (*APC*) gene are the most common disease-causing genetic events in humans; approximately 50% of the population will develop colorectal polyps initiated by such mutations during a normal life span (14). Individuals who inherit *APC* mutations develop thousands of colorectal tumors, consistent with *APC*'s tumor suppressor or "gatekeeping" role in colorectal tumorigenesis (15,16). *APC* homodimerizes through its amino-terminus (17), and interacts with at least six other proteins: β -catenin (18), γ -catenin (plakoglobin) (19), tubulin (20), EB1 (21), hDLG, a homologue of a *Drosophila* tumor suppressor protein (22), and ZW3/GSK3 β kinase (23). Whether any of these interacting proteins communicate *APC*

growth-controlling signals is unknown. Thus there is a need in the art for a fuller understanding of how the tumor suppressor gene APC functions in cells.

SUMMARY OF THE INVENTION

5 It is an object of the present invention to provide human nucleotide sequences encoding transcriptional activation proteins.

It is another object of the present invention to provide isolated preparations of transcriptional activation proteins.

10 It is an object of the present invention to provide methods of determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway.

It is an object of the present invention to provide a fusion protein useful for binding to β -catenin.

It is an object of the invention to provide a polypeptide useful for binding to β -catenin.

15 It is an object of the invention to provide a method for inhibiting the growth of a tumor cell.

It is an object of the invention to provide a method for inducing apoptosis of a tumor cell.

20 It is an object of the invention to provide an adenovirus vector useful for binding to β -catenin.

Another object of the invention is to provide methods of identifying candidate drugs for use in Familial Adenomatous Polyposis (FAP) patients or patients with increased risk of developing cancer.

25 It is yet another object of the invention to provide methods of identifying candidate drugs for the treatment of cancer patients, in particular those with *APC* or β -catenin mutations.

Another object of the invention is to provide a method for diagnosing cancer in a sample suspected of being neoplastic.

Another object of the invention is to provide a method for treating a patient with colorectal cancer or other cancer associated with FAP.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment of the invention an intron-free DNA molecule is provided which encodes Tcf-4
5 protein as shown in SEQ ID NO: 2 or 4.

According to another embodiment of the invention an isolated Tcf-4 protein is provided. The protein is substantially free of other human proteins, and has a sequence as shown in SEQ ID NO: 2 or 4.

10 In another embodiment of the invention a method is provided for determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway. The method comprises the steps of:

introducing a Tcf-responsive reporter gene into the cell; and
15 measuring transcription of said reporter gene; wherein a cell which supports active transcription of said reporter gene does not have wild-type APC or does not have a wild-type downstream protein in the APC transcription regulatory pathway.

According to yet another embodiment of the invention a method is
20 provided for determining the presence or absence in a cell of wild-type APC. The method comprises the steps of:

contacting a Tcf-responsive reporter gene with a lysate of the cell; and

measuring transcription of said reporter gene; wherein a lysate
25 which inhibits said transcription has wild-type APC.

In still another embodiment of the invention a method of identifying candidate drugs is provided. The drugs may be useful for treatment of FAP or other cancer patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a cell having no wild-type APC or a mutant β -catenin with a test compound;

measuring transcription of a Tcf-responsive reporter gene, wherein a test compound which inhibits the transcription of the reporter gene is a candidate drug for cancer therapy.

According to yet another aspect of the invention another method is provided for identifying candidate drugs for use in for use in FAP patients, colon cancer patients, patients with mutations in β -catenin or *APC*, or patients with increased risk of developing cancer. The method, comprises the steps of:

contacting a Tcf-responsive reporter gene with a test compound under conditions in which the reporter gene is transcribed in the absence of the test compound; and

measuring transcription of the Tcf-responsive reporter gene; wherein a test compound which inhibits said transcription is a candidate drug for cancer therapy.

According to another aspect of the invention a method is provided for identifying candidate drugs for use in FAP patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a test compound with β -catenin and Tcf-4 under conditions in which β -catenin and Tcf-4 bind to each other; and

determining whether the test compound inhibits the binding of β -catenin and Tcf-4, a test compound which inhibits the binding being a candidate for cancer therapy or prophylaxis.

According to still another embodiment of the invention a method is provided for diagnosing cancer in a sample suspected of being neoplastic. the method comprises the steps of:

comparing a CTNNB sequence found in the sample to a second CTNNB sequence found in a normal tissue, wherein a difference between the first and second sequence is an indicator of cancer.

According to one aspect of the invention a fusion protein is provided. It comprises an enzyme covalently linked to a portion of APC which comprises its β -catenin binding domain. A polynucleotide encoding the fusion protein is also provided.

5 According to another embodiment a polypeptide is provided. The polypeptide comprises a portion of APC which comprises its β -catenin binding domain. An isolated and purified polynucleotide encoding the polypeptide is also provided.

10 According to another aspect of the invention a method is provided for inhibiting the growth of a tumor cell. A polynucleotide which encodes a polypeptide comprising the β -catenin binding domain of APC is administered to the tumor cell, whereby growth of the tumor cell is inhibited.

15 According to another aspect of the invention a method is provided for inducing apoptosis of a tumor cell. A polynucleotide which encodes a polypeptide comprising the β -catenin binding domain of APC is administered to the tumor cell, whereby apoptosis of tumor cells is induced.

Also provided by the present invention is an adenovirus vector which comprises a polynucleotide encoding the β -catenin binding domain of APC.

20 According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP. The method comprises the step of:

administering to the patient a nucleotide sequence comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

25 According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP. The method comprises the step of:

30 administering to the patient a polypeptide comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

The present invention thus provides the art with diagnostic, therapeutic and drug discovery methods especially useful for FAP and other cancers with *APC* or β -catenin mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Sequence comparison of hTcf-4 and hTcf-1.

Two alternative splice forms of *hTcf-4* were identified, each encoding a different COOH-terminus. One form (hTcf-4E; SEQ ID NO:6) was homologous to hTCF-1E (Fig. 1A) (7); the other form (hTcf-4B; SEQ ID NO: 5) was homologous to hTcf-1B (Fig. 1B). The highly conserved NH₂-terminal interaction domain and the High Mobility Group (HMG) box DNA-binding region are boxed. Abbreviations for the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; IC, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; P, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence has been deposited in GenBank (accession number:)

Figs. 2A, 2B, and 2C. Analysis of *hTcf-4* expression in colonic epithelium.

(Fig. 2A) Northern blot analysis of *hTcf-4*, *hTcf-1*, *hLef-1* expression in Jurkat T cells (lane 1); colonic mucosa (lane 2); colon carcinoma cell lines DLD-1 (lane 3), HCT116 (lane 4); SW480 (lane 5); SW620 (lane 6); HT29 (lane 7). Lane 2 contains 5 μ g total RNA; all others contain 15 μ g total RNA. The positions of 18S and 28S ribosomal RNAs are shown. EtBr, ethidium bromide stain. (Fig. 2B) In situ hybridization of healthy human colon tissue to an *hTcf-4* probe. (Fig. 2C) In situ hybridization to a negative control probe (a fragment of the *E. coli* neomycin resistance gene).

Figs. 3A, 3B. Transactivational properties of β -catenin/hTcf-4.

All reporter assays were performed as duplicate transfections. For each condition, both values are shown. **(Fig. 3A)** Reporter gene assays in IIA1.6 B cells. Cells were transfected by electroporation with 1 μ g luciferase reporter plasmid, 5 μ g β -catenin expression plasmid, and 3 II- hTcf-4 expression plasmids. Empty pCDNA was added to a total of 10 μ g, plasmid DNA. **(Fig. 3B)** Reporter gene assays in SW480 colon carcinoma cells. Cells were transfected with 0.3 μ g, of the indicated luciferase reporter gene, 0.7 μ g pCATCONTROL as internal control, the indicated amounts of pCMVNeo-APC, and empty PCDNA to a total of 2.5 μ g plasmid DNA. Control CAT values are given in the right panel.

Fig. 4. Constitutive presence of β -catenin-hTcf-4 complexes in APC^{-/-} cells. Gel retardation assays were performed on nuclear extracts from the indicated cell lines before and after a 20-hour exposure to Zn⁺⁺. Samples in lanes 1, 4, 7, 10 were incubated under standard conditions. To the samples in lanes 2, 5, 8, 11, 0.25 μ g, anti β -catenin was added. To the samples in lanes 3, 6, 9, 12, 0.25 μ g of a control (human CD4) antibody was added. N.S., nonspecific band also observed with mutant (nonbinding) probe (lane Mt).

Figs. 5A and 5B. Effects of APC mutations on catenin regulated transcription (CRT). **(Fig. 5A)** Schematics of wild-type (WT) and mutant APC. APC is a 2843-amino-acid (AA) protein (32) with contains armadillo (ARM) repeats in the amino-terminus (33), 15 and 20 AA β -catenin-binding repeats in the central region (18,19), and a basic region in the carboxyl-terminus (32). The carboxyl-terminus also contains a TXV sequence which mediates DLG binding (22). **(Fig. 5B)** Effects of WT and mutant APC on CRT. SW480 cells containing endogenous mutant APC were transfected with the APC expression vectors shown in **(Fig. 5A)** and CRT was measured. Cells were transfected with increasing amounts of WT APC (0, 0.15 and 0.5 μ g) or 0.5 μ g mutant APC. CRT reporter activities are

expressed relative to assays containing no WT APC and are the means of three replicates. Error bars represent standard deviations.

Lipofectamine was used to cotransfect SW480 cells with an internal control (0.5 μ g pCMV- β gal), a reporter construct (0.5 μ g pTOPFLASH or pFOPFLASH) and the indicated amount of the various APC expression vectors. The pTOPFLASH reporter contained an optimized Tcf-binding site 5' of a luciferase reporter gene, whereas pFOPFLASH contained a mutated site that does not bind Tcf. The amount of DNA in each transfection was kept constant by addition of an appropriate amount of empty expression vector (pCEP4). Luciferase and β -galactosidase activities were determined 16 hours after transfection. Luciferase activity was corrected for transfection efficiency (using the control β -galactosidase activity) and nonspecific transcription (using the pFOPFLASH control).

Figs. 6A and 6B. Evaluation of CRT in colorectal cancer cell lines with WT APC. **(Fig. 6A)** Immunoblot of endogenous APC in the DLD1, SW480, HCT116, SW48 and 293 cell lines, developed with APC monoclonal antibody FE9 (34). **(Fig. 6B)** Effects of exogenous WT APC on CRT in cell lines with endogenous mutated or WT APC. Cells were transfected with increasing amounts (0, 0.15 μ g, 0.5 μ g for DLD1 and SW48; 0, 0.5 μ g, 5 μ g for HCT116) of WT APC or APC1309 Δ mutant (0.5 μ g for DLD1 and SW48; 5 μ g for HCT116) and CRT was assessed as in Fig. 5. CRT reporter activities are expressed relative to activity in extracts without exogenous APC and are the means of three replicates. Error bars represent standard deviations.

Figs. 7A, 7B, and 7C. Evaluation of β -catenin in colorectal cancer cell lines with WT APC. **(Fig. 7A)** Immunoblot of the cell lines used in this study, developed with β -catenin monoclonal C19220 (Transduction Laboratories, Lexington, KY)(31). **(Fig. 7B)** Sequence of *CTNNB1* in HCT116 and SW48. Overlapping segments constituting the entire *CTNNB1* were amplified by RT-PCR from SW480, DLD1, HCT116, and SW48 cells, and sequenced directly with ThermoSequenase (Amersham). In the case of

HCT116, a PCR product containing the deleted region was also cloned into pCI-neo (Promega, Madison) and multiple clones corresponding to each allele were individually sequenced.

The left panel (nts 121 to 143 from HCT116) reveals the presence of a deletion in addition to the WT sequence. The middle panel (antisense strand 156 to 113 of the WT and deleted alleles of HCT116) reveals the 3-bp deletion (Δ TCT) that removed codon 45 in half the clones. The right panel (nts 80 to 113 from SW48) reveals a C to A transition affecting codon 33 (TCT to TAT). (Fig. 7C) Schematic of β -catenin illustrating the armadillo repeats (33) in human (SEQ ID NO: 10), xenopus (SEQ ID NO: 10), and drosophila (SEQ ID NO: 11), and negative regulatory domain. The residues in larger type fit the consensus sequence for GSK3 β phosphorylation (29) and those in bold have been demonstrated to affect down regulation of β -catenin through GSK3 β phosphorylation in *Xenopus* embryos (27). The five mutations found in human colon cancers are indicated at the top.

Figs. 8A and 8B. Functional evaluation of β -catenin mutants. (Fig. 8A) Constitutive nuclear complex of β -catenin and Tcf in HCT116 cells. The presence of nuclear β -catenin-Tcf complexes was assessed by gel shift assays. Lanes 1 to 3, optimal Tcf retardation probe shifted with nuclear extract from HCT116 cells with addition of no antibody (lane 1), anti β -catenin (0.25 μ g, lane 2), or an irrelevant antibody (0.25 μ g, lane 3). Lane 4, mutant Tcf retardation probe shifted with nuclear extract from HCT116 cells. n.s., nonspecific shifting seen with the mutant probe. (Fig. 8B) Effects of the β -catenin mutations on CRT. 293 cells were transfected with WT (WT) or mutant (Δ 45, S33Y) β -catenin and CRT was assessed. CRT reporter activities are expressed relative to WT β -catenin and are the means of three replicates. Error bars represent standard deviations. β -catenin expression constructs were prepared as follows. WT *CTNNB1* was amplified by RT-PCR from SW480 cells and cloned into the mammalian expression vector pCI-neo

(Promega) to produce pCI-neo- β -cat. The pCI-neo- β -cat $\Delta 45$ and S33Y were generated by replacing codons 1 to 89 in pCI-neo- β -cat with a PCR product encoding the equivalent region from HCT116 or SW48 cDNA, respectively. The structures of all constructs were verified by sequence analysis.

5 Lipofectamine was used to cotransfect 293 cells with an internal control (0.1 μ g CMV- β gal), a reporter (0.5 μ g pTOPFLASH or pFOPFLASH), a Tcf-4 expression vector (0.5 μ g pCDNA-TCF4), and β -catenin (0.5 μ g) or dominant negative hTcf-4 1.0 μ g) expression vectors. CRT was determined as described above.

10 **Figs. 9A, 9B and 9C.** Characterization of the Ad-Mini-ME. **Fig. 9A.** Map of GFP/cAPC incorporated into Ad-Mini-ME. Linear representation of different domains in APC including oligomerization domains, armadillo repeats, 15-aa repeats, 20-aa repeats, basic domain, and EB-1 binding site are shown. The β -catenin binding and degradation domain which comprises 15-aa

15 repeats, 20-aa repeats and SAMP repeats was fused with the carboxyl-terminal of GFP. Expression of this cassette is driven by a CMV promoter in Ad-Mini-ME adenovirus vector. **Fig. 9B.** Western blot analysis with an anti-GFP antibody. Infection of SW480 cells with Ad-Mini-ME resulted in production of a fusion protein of the expected size (150 kD) whereas the

20 Ad-GFP infected cells generated the expected 17 kD GFP. **Fig. 9C.** Fluorescence microscopy revealed that the GFP/cAPC (green fluorescence protein/central region of APC) fusion protein was diffusely localized to the cytoplasm in DLD1 and HCT116 cells.

Figs. 10A and 10B. Ad-Mini-ME suppresses CRT. **Fig. 10A.** The

25 luciferase activity was dramatically inhibited in Ad-Mini-ME infected DOT cells as compared with control and Ad-GFP infected DOT cells. **Fig. 10B.** No significant differences in luciferase activity among control, Ad-GFP infected, and Ad-Mini-ME infected Dluc cells were observed at day 1 and 2. Values are the average of three experiments.

Fig. 11. Western blot analysis of c-MYC and cyclin D1 in DLD1, SW480, and HCT116. As compared with the control and Ad-GFP infected cells, c-MYC expression is strongly repressed in both Ad-Mini-ME infected DLD1 and SW480 cells but is only partially inhibited in Ad-Mini-ME infected HCT116. Similarly, repression of cyclin D1 expression by Ad-Mini-ME is observed in DLD1 and SW480 but not in HCT116. Similar expression levels of α -tubulin in each lane indicate that similar amounts of protein are loaded.

Figs. 12A and 12B. Immunofluorescence staining of β -catenin in DLD1 (Fig. 12A) and SW480 (Fig. 12B).

Cells were infected with Ad-Mini-ME or Ad-GFP as indicated. Cells were stained for β -catenin and counterstained with DAPI as indicated. Ad-GFP infected cells demonstrate a predominant nuclear staining of β -catenin. In contrast, almost all the Ad-Mini-ME infected cells exhibited cytoplasmic and membrane β -catenin staining with minimal nuclear staining.

Figs. 13A and 13B. Cell growth and colony formation assays. **Fig. 13A.** Growth kinetics in DLD1, SW480, and HCT116 cells after mock-, Ad-GFP- or Ad-Mini-ME infections, as indicated. **Fig. 13B.** Colony formation in collagen gel after mock-, Ad-GFP- or Ad-Mini-ME infections, as indicated. The number of colonies is indicated below each well.

Fig. 14. Apoptosis in virus-infected cells. Cells were stained with Hoechst33258 dye and at least 500 cells were counted and the results are expressed as the fold increase in the percentage of apoptotic cells in each sample.

Fig. 15. Proliferation and apoptosis in DLD1 induced tumors. Subcutaneous tumors in nude mice were generated by injecting Ad-Mini-ME or Ad-GFP infected DLD cells as indicated. Hematoxylen

and eosin (H&E) staining revealed extensive apoptosis in Ad-Mini-ME infected cells. Immunoperoxidase staining with the M30 antibody (M30) which recognizes apoptotic cells revealed that the majority of Ad-Mini-ME infected cells were positive while only a few Ad-GFP infected cells were positive for this antibody. Immunoperoxidase staining with an antibody specific for the proliferation marker Ki-67 (Ki-67) revealed a remarkable decrease in Ki-67 nuclear labeling in Ad-Mini-ME infected cells. In contrast, the Ad-GFP infected cells were diffusely positive.

DETAILED DESCRIPTION

It is a discovery of the present invention that hTcf-4 binds to β -catenin and activates transcription in colorectal epithelial cells. Moreover, it has now been found that APC regulates this transcriptional activation, at least in part by binding to β -catenin. In colorectal cancer cells this regulation is frequently abrogated, either by mutation of APC or by mutation of β -catenin.

Two alternative splice forms of human Tcf-4 have been found. One form (hTcf-4E) is homologous to hTcf-1E and the other (hTcf-4B) is homologous to hTcf-1B. The sequence of the nucleotide and amino acid sequences are shown in SEQ ID NOs: 1-4. The coding sequences and proteins can be used in assays as described below. Intron-free DNA molecules are provided which are originally made by reverse transcription of a mRNA molecule. They can be propagated in cells or amplified as is desired. Isolated Tcf-4 proteins can be provided substantially free of other human proteins if, for example, the nucleotide sequences are expressed in non-human cells. Methods and vectors for achieving such expression are well known in the art. Choice of such expression means is made by the skilled artisan according to the desired usage and convenience.

Cells can be tested to determine if they have a wild-type APC or a wild-type downstream protein in the APC transcription regulatory pathway, called

herein the CRT pathway (β -catenin/Tcf-regulated transcription). One protein within the CRT pathway which has been identified as a target of mutations in human cancers is β -catenin (encoded by the *CTNNB1* gene). Other parts of the pathway are also likely to be targets. Although the target genes of the CRT pathway have not been identified, they can be readily identified using the system disclosed here. Genes which are differentially transcribed in the presence of wild-type and mutant *CTNNB1*, for example, can be identified.

Tcf-responsive reporter genes are those constructs which comprise a readily detectable or assayable gene (such as luciferase, β -galactosidase, chloramphenicol acetyltransferase) linked *in cis* to a Tcf-responsive element. Such responsive elements are known in the art (7) and any such elements can be used. An optimal Tcf motif contains the sequence CCTTTGATC. From one to twenty copies, and preferably from three to six copies, of the motif may be used. Mutation of the sequence to CCTTTGGCC abrogates responsiveness. Another necessary part of such constructs is a minimal promoter, such as the *c-Fos* or the Herpes virus thymidine kinase promoter. Transcription of the reporter gene may be performed by any means known in the art, usually by assaying for the activity of the encoded gene, although immunological detection methods can also be used. In addition, transcription can be monitored by measuring the transcribed mRNA directly, typically using oligonucleotide probes.

As shown below, a cell which has a wild-type APC protein will inhibit CRT. However, most mutations in *APC* render APC unable to inhibit CRT. Similarly, certain mutations in *CTNNB1* render β -catenin super-active and/or refractory to the inhibition by APC. Thus measuring Tcf-responsive reporter gene transcription is an indication of the status of *APC* and *CTNNB1*. Mutations in both of these genes are associated with cancers and therefore provides diagnostic and prognostic information.

Assays for CRT can be accomplished *in vitro* or in cells. If the assay is to be accomplished in cells, then a Tcf-responsive reporter gene must be introduced into the cell. Any means for introducing genetic material into cells can be used, including but not limited to infection, transfection, electroporation. If the assay is to be performed *in vitro* then the components for transcription must be present. These include suitable buffers, RNA polymerase, as well as ribonucleotides. If the protein product is to be assayed, then the components for translation must also be present, such as ribosomes, and amino acids.

These assays can also be used to screen compounds for potential as anti-cancer therapeutic agents. Using either the *in vitro* or cell form of the assay, test compounds can be introduced to determine whether they are able to mimic the effect of wild-type APC or to convert a mutant APC into a form which is able to inhibit CRT or a mutant β -catenin into a form which is regulatable by APC. In addition, compounds can be tested for the ability to inhibit the binding of β -catenin and Tcf-4, thus mimicking the action of APC. Such a test can be conducted *in vitro* or *in vivo*, for example using a two hybrid assay.

A means for diagnosis of cancers is the result of the observation that *CTNNB1* mutations are found in tumor cells, especially those which have wild-type APC. Such mutations can be found, *inter alia*, by sequencing either the gene or the protein found in a sample. Functional assays can also be used, such as whether β -catenin binds to APC or Tcf-4, or whether it is capable of mediating CRT. Sequences can be compared to those found in a normal tissue of a human, especially the same human who provided the sample being tested. Suitable tumors for testing include, but are not limited to those which are associated with FAP. Suitable tumors include colorectal cancer, thyroid cancer, brain cancer, medulloblastoma, desmoid tumor, osteoma, breast cancer, and head and neck cancer. Because APC mutations

are so frequent, and because it appears that APC mutations do not occur in the same tumors as CTNNB1 mutations, one can prescreen samples for APC mutations before performing a CTNNB1 determination.

Fusion proteins are known in the art and are the product of recombinant technology. At least two polypeptides or proteins which derive from different naturally occurring proteins are joined together as a single polyamino acid sequence. Typically this is accomplished by engineering two polynucleotides which encode the polypeptides or proteins so that they form a single message-encoding unit which contains a single open reading frame. One of the polypeptides can be an enzyme or other readily detectable protein. One of the polypeptides is a portion of APC which comprises the β -catenin binding site. The APC-derived polypeptide may be the N-terminal or the C-terminal portion of the fusion protein, or if more than two polypeptides are joined together, the APC-derived polypeptide can be internal. One particularly useful fusion partner is green fluorescent protein (GFP) which is readily detectable. Other useful enzymes, such as luciferase, β -galactosidase, and chloramphenicol acetyltransferase can also be used as is convenient. Other polypeptide moieties, such as epitope tags, can also be engineered into the fusion protein. These can be used, for example, to facilitate detection and purification of the fusion protein. The fusion protein is advantageous because it permits an independent means of verification that the APC-derived polypeptide is being expressed in the cell. However, if such verification is not desired or required, a non-fused polypeptide portion of APC can be used. Such portion must be functional in binding to β -catenin. Preferably the portion will not contain at least one of the following domains: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain. These domains are apparently not required to mediate growth inhibition and apoptosis.

Polynucleotides which encode the APC-derived portion, whether in fusion protein form or as a polypeptide, can be regulated using any of the promoters, transcription terminators, enhancers, and transregulators known in the art. Expression can be regulated or constitutive as is desired. The regulation may be by an exogenous agent, such as a drug which can be administered, or by endogenous agents, such as developmentally or environmentally regulated factors. The polynucleotide may be isolated and purified away from other molecules, and in particular isolated and purified from full-length APC-encoding polynucleotides. Methods for isolating and purifying polynucleotides of known sequence and/or size are known in the art, and any such method can be used. Any vector known in the art can be used, including viral vectors, plasmids, mini-chromosomes, and yeast artificial chromosomes. One particularly preferred vector is an adenovirus vector.

Any method known in the art can be used for administering polynucleotides to tumor cells or subjects. These include without limitation, transfection, liposomes, viral infection, particle bombardment, nanoparticles, microparticles, compacted nucleic acid complexes, intravenous, intradermal, subcutaneous, intramuscular, direct injection into a tumor. Combinations of these methods may also be used.

The portion of the APC gene which encodes the β -catenin binding site can be used in a gene delivery format. This portion comprises approximately amino acid residues 958 to 2075, although fewer residues may be sufficient to bind to β -catenin. For example, residues 1000-2000, 1100-1900, 1200-1800, or 1300-1700, may be sufficient to bind to β -catenin. Suitable techniques are known in the art for administering genes to tumors and tumor cells, and any such technique can be used. Suitable expression vectors are also known in the art and it is within the skill of the artisan to select an appropriate one. Upon expression in a tumor cell of the β -catenin binding portion of APC, β -catenin will be bound and titrated away from binding to

Tcf-4, thus preventing unregulated expression of the CRT target genes. Similarly, a polypeptide portion of APC containing the β -catenin binding site can be administered to cells to perform a titration of β -catenin. Techniques for such administration to cells is well known in the art. Cells which are
5 treated with either the polynucleotide or the polypeptide can be used to study the interaction between APC and β -catenin, and for developing drugs which interfere with such binding. Such treatment can also induce apoptosis and inhibit cell growth.

Polynucleotides may be administered to cells, subjects, or patients
10 according to the present invention for the purpose of screening for agents which enhance polynucleotide transfer to cells or enhance subsequent biological effects of the polynucleotides or their encoded proteins. Such biological effects include expression of the polynucleotide to form mRNA and/or protein, replication of the polynucleotide, hybridization to
15 complementary messenger RNA and inhibition of its translation, and integration of the polynucleotide. According to the present invention, expression of a polypeptide or fusion protein encoded by the administered polynucleotide is preferred so that the polypeptide or fusion protein can bind to β -catenin. The result of such binding is inhibition of tumor cell growth and/or induction of apoptosis. Agents can also be screened for the ability to
20 enhance the binding of the encoded polypeptide or fusion protein to β -catenin. Agents which can be screened include any test compounds or substances, whether natural products or synthetic, which can be administered to the cells, subject, or patient. Libraries or mixtures of compounds can be tested. The
25 compounds or substances may be those for which a pharmaceutical effect is previously known or unknown. The compounds or substances may be delivered before, after, or concomitantly with the polynucleotides. They may be administered separately or in admixture with the polynucleotides. Integration of delivered DNA can be monitored by any means known in the

art. For example, Southern blotting of the delivered DNA can be performed. A change in the size of the fragments of the delivered polynucleotide indicates integration. Replication of the delivered polynucleotide can be monitored *inter alia* by monitoring incorporation of labeled nucleotides combined with hybridization to a probe for the delivered polynucleotides. Expression of the polynucleotide can be monitored by detecting production of RNA which hybridizes to the delivered polynucleotide or by detecting protein encoded by the delivered polynucleotide. The protein can be detected immunologically or by activity, for example. The expression of the protein can also be monitored by detecting the effects of β -catenin binding. Thus the administration of the polynucleotides according to the present invention provides an excellent system for screening agents for their ability to promote delivery, integration, hybridization, expression, replication or integration in cells or in an animal, preferably a mammal, more preferably a human.

Tumor cell inhibition can be measured or assessed according to any method known in the art. Tumor mass may be observed and measured. Time until palpable tumor development after administration of tumor cells to a nude mouse can be assessed. Survival time of animals with tumors can be measured. Incorporation of tritiated thymidine can be measured. Focus formation can be measured. Any method known in the art can be used, without limitation. Monitoring of apoptosis can also be done as is known in the art. DNA fragmentation, a hallmark of apoptosis, can be assayed by any means known in the art, including but not limited to electrophoresis on a gel, staining nuclei with TUNEL, and photometric enzyme immunoassay for the determination of cytoplasmic histone-associated DNA fragments. The assays can be performed *in vitro* or *in vivo*.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific

examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

This example identifies Tcf-4 as the expressed family member in colorectal epithelial cells and provides the complete sequence of the cloned cDNA.

There are four known members of the Tcf/Lef family in mammals: the lymphoid-specific factors Tcf-1 and Lef-1 (7,8), and the less well characterized Tcf-3 and 4(9). We performed a qualitative Reverse Transcriptase-Polymerase Chain Reaction assay for expression of the four *Tcf/Lef* genes on 43 colon tumor cell lines. While most colon cell lines expressed more than one of the genes, only hTcf-4 mRNA was expressed in essentially all lines.

We then screened a human fetal CDNA library and retrieved clones encoding full-length hTcf-4 (Fig. 1). A genomic fragment encoding, the HMG box region of hTcf-4 (7) was used to probe a human 12 week-fetal cDNA library in Lambda GT-11. Positive clones were subcloned into pBluescriptSK and sequenced. See SEQ ID NOs: 1 and 3. The predicted sequence of hTcf-4 was most similar to that of hTcf-1. Alternative splicing yielded two COOH-termini that were conserved between hTcf-1 and hTcf-4. The NH₂-terminus, which in hTcf-1, mLef-1 and *Xenopus* TCF-3 mediates binding to β -catenin (6), was also conserved in hTcf-4. Northern blot analysis of selected colon carcinoma cell lines revealed high-level expression of *hTcf-4* (Fig. 2A). Northern blot hybridizations (7) were performed with full-length *hTcf-1*, *hLef-1* and *hTcf-4* cDNA. Colon epithelial cells were freshly prepared from a mucosal preparation dissected from a healthy surgical colon sample. The sample was minced, and incubated with 1 mM dithiothreitol (DTT) in Hanks' medium to remove mucus. Single-cell suspensions were prepared by

incubation at RT in 0.75 mM EDTA in Hanks' medium. Epithelial cells were separated from lymphocytes by Percoll gradient centrifugation.

As evidenced by in situ hybridization (Fig. 2, B and C) and Northern blotting (Fig. 2A), *hTcf-4* mRNA was readily detectable in normal colonic epithelium, whereas *hTcf-I* and *hLef-I* were not detectable. In situ hybridization of 6 μ frozen sections of healthy colon biopsy samples were performed as described(10). *hTcf-4 cDNA* encoding amino acids 200 to 310 was amplified and labeled with Dig-11-dUTP (Boehringer Mannheim, Germany) by PCR. After hybridization and washing, the sections were sequentially incubated with mouse anti-Dig antibody (Boehringer) and a horseradish peroxidase conjugated rabbit antibody to mouse immunoglobulin (Dako, Glostrup, Denmark). The signal was visualized with diaminobenzidine, which produces a reddish-brown precipitate. Blue counterstaining was performed with hematoxylin.

EXAMPLE 2

This example demonstrates the interaction of Tcf-4 and β -catenin and their function as a transcriptional activating factor.

To investigate whether hTcf-4 functionally interacts with β -catenin, we used two sets of reporter constructs in a β -catenin-Tcf reporter gene assay (7). One contained three copies of the optimal Tcf motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal *c-Fos* promoter driven-luciferase expression (PTOPFLASH and PFOPFLASH). The second set contained three copies of the optimal motif, or three copies of the mutant motif, upstream of a minimal Herpes virus thymidine kinase promoter driven-Chloramphenicol Acetyl Transferase (CAT) expression (PTOPCAT and PFOPCAT, respectively). Reporter gene assays were performed as in (7). In brief, 2×10^6 cells were transfected with plasmids by electroporation. After 24 hours, cells were harvested and lysed in 1 mM DTT, 1 % Triton X-

100, 15 % glycerol, 25 mM Tris pH 7.8 and 8 mM MgCl₂. cDNAs encoding Myc-tagged versions of β -catenin and hTcf-4 were inserted into the mammalian expression vector pCDNA (Invitrogen). PCATCONTROL, encoding the CAT enzyme under the control of the SV40 promoter, was purchased from Promega.

Epitope-tagged hTcf-4 and a deletion mutant lacking the NH₂-terminal 30 amino acids (Δ NhTcf-4) were cloned into the expression vector pCDNA. Transient transfections were performed in a murine B cell line (IIA1.6), that does not express any of the Tcf genes (6).

The TOPFLASH reporter was strongly transcribed upon cotransfection with the combination of β -catenin and hTcf-4 plasmids, but not with the individual plasmids or with the combination of β -catenin and Δ NhTcf-4 plasmids. No enhanced transcription was detected in cells transfected with the negative control PFOPFLASH (Fig. 3A). These results show that interaction of the NH₂-terminus of hTcf-4 with β -catenin results in transcriptional activation.

EXAMPLE 3

This example demonstrates the functional regulation of CRT transcriptional activation by wild-type APC.

In three APC^{-/-} carcinoma cell lines, SW480, SW620 and DLD-1 (Fig. 3B), the PTOPFLASH reporter was 5-20 fold more actively transcribed than PFOPFLASH. Importantly, transfection of SW480 cells with the reporter gene and an APC-expression vector abrogated the transcriptional activity in a dose-dependent manner (Fig. 3B). In contrast APC had no effect on a cotransfected internal control (pCATCONTROL), or on the basal transcription of PFOPFLASH (Fig. 3B). The use of PTOPCAT and PFOPCAT instead of PTOPFLASH and PFOPFLASH led to comparable observations. The constitutive transcriptional activity of Tcf reporter genes in APC^{-/-} colon carcinoma cells was in stark contrast to the inactivity of these genes in non-

colonic cell lines, including IIA1.6 B cells (Fig. 3A), the C57MG breast carcinoma cell line; the Jurkat and BW5147 T cell lines; the Daudi and NS1 B cell lines; the K562 erythromyeloid cell line; the HeLa cervical carcinoma line; the HepG2 hepatoma cell line; 3T3, 3T6, and Rat-I fibroblasts; and the
5 kidney derived SV40-transformed COS cell line (7,16).

EXAMPLE 4

This example demonstrates that a functional β -catenin-hTcf-4 complex exists constitutively in APC^{-/-} cells.

We used HT29-APC^{-/-} colon carcinoma cells (12), in which APC is
10 controlled by a metallothionein promoter. Induction by Zn⁺⁺ restores wild-type levels of APC, and leads to apoptosis (12). HT29-Gal cells which carry a Zn⁺⁺-inducible LacZ gene were used as a control. The only Tcf family member expressed in HT29 is hTcf-4 (Fig. 2C). In nuclear extracts from uninduced HT29 derived transfectants, we readily detected hTcf-4 by gel
15 retardation (Fig. 4). An additional band of slightly slower mobility was also observed. The addition of a β -catenin antibody resulted in the specific retardation of the latter band, indicating that it represented a β -catenin-hTcf-4 complex (Fig. 4) (12). After Zn⁺⁺ induction for 20 hours, the β -catenin-hTcf-4 complex was diminished sixfold relative to uncomplexed hTcf-4 in
20 HT29-APC1, while no significant change was observed in HT29-Gal cells (Fig. 4). Importantly, the overall levels of cellular β -catenin do not change during the induction period in HT29-APC1 cells (12).

Gel retardation assays were performed as described elsewhere (7). Extracts were prepared from intact nuclei that were washed four times to
25 avoid contamination with cytoplasmic β -catenin. As the optimal Tcf/Lef probe, we used a double-stranded 15-mer CCCTTTGATCTTACC (SEQ ID NO: 3); the control probe was CCCTTTGGCCTTACC (SEQ ID NO: 4). (All oligonucleotides were from Isogene, Holland). The β -catenin antibody was purchased from Transduction Laboratories (Lexington, KY). A typical

binding reaction contained 3 μ g nuclear protein, 0.1 ng radiolabeled probe, 100 ng of dIdC, in 25 μ l of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). Samples were incubated for 20 min at room temperature, antibody was added, and the samples incubated 20 min further.

5 On the basis of these data, we propose the following model. In normal colonic epithelium hTcf-4 is the only expressed member of the Tcf family. The interaction of β -catenin with hTcf-4 is regulated by APC. When appropriate extracellular signals are delivered to an epithelial cell, β -catenin accumulates in a form that is not complexed with GSK3 β -APC, and that
10 enables its nuclear transport and association with hTcf-4. The HMG domain of hTcf-4 binds in a sequence-specific fashion to the regulatory sequences of specific target genes; β -catenin supplies a transactivation domain. Thus, transcriptional activation of target genes occurs only when hTcf-4 is associated with β -catenin. The hTcf-4 target genes remain to be identified.
15 However, the link with APC and catenin suggests that these genes may participate in the generation and turnover of epithelial cells. Upon loss of wild-type APC, monomeric β -catenin accumulates in the absence of extracellular stimuli, leading to uncontrolled transcription of the hTcf-4 target genes. The apparent *de novo* expression of other members of the *Tcf* family
20 in some colon carcinoma cell lines might lead to a further deregulation of Tcf target gene expression by the same mechanism. The control of β -catenin -Tcf signaling is likely to be an important part of the gatekeeper function of APC (19), and its disruption an early step in malignant transformation.

EXAMPLE 5

25 This example demonstrates that mutant APC protein does not regulate CRT and that a complete set of 20-AA repeats in APC is required to mediate inhibition of CRT.

We tested four APC mutants (Fig. 5A) for their ability to inhibit β -catenin/Tcf-regulated transcription (CRT) in transfection assays. The first mutant, APC331 Δ represents a type of mutation found in the germline of Familial Adenomatous Polyposis (FAP) patients as well as in sporadic tumors (15). The APC331 Δ protein is truncated at codon 331, amino-terminal to the three 15-amino-acid (AA) β -catenin-binding repeats between codons 1020 and 1169. The second mutant, APC1309 Δ , is the most common germline *APC* mutation (15), a 5-bp deletion that produces a frameshift at codon 1309 and truncation of the protein. The APC1309 Δ protein retains the 15-AA β -catenin repeats but lacks the seven 20-AA repeats between codons 1323 and 2075 that have been implicated in binding and phosphorylation of β -catenin (18). The third mutant, APC1941 Δ , represents one of the most distal somatic mutations observed in colorectal tumors (25). The APC1941 Δ protein is truncated at codon 1941 and therefore contains the 15-AA repeats and all but the last two 20-AA repeats. Finally, APC2644 Δ represents a germline mutation resulting from a 4-bp deletion in codon 2644. Patients with this type of unusual carboxyl-terminal mutation develop few polyps (attenuated polyposis) but have pronounced extracolonic disease, particularly desmoid tumors (26).

Each of the *APC* mutants was cotransfected with a CRT reporter into the SW480 colorectal cancer cell line. SW480 cells have truncated APC and constitutively active CRT which can be suppressed by exogenous WT APC. Although all four mutants produced comparable levels of APC protein after transfection, they varied in their CRT inhibitory activity. The three mutants found in patients with typical polyposis or cancer were markedly deficient in inhibition of CRT (Fig. 5B). The reduced activity of APC1309 Δ and APC1941 Δ suggests that β -catenin binding is not sufficient for APC-mediated inhibition of CRT and that the complete set of 20-AA repeats is required. Interestingly, the inhibitory activity of the APC2644 Δ mutant associated with attenuated polyposis was comparable to that of WT APC (Fig. 5B), suggesting

that the DLG-binding domain at the carboxyl-terminus of APC is not required for down-regulation of CRT.

WT and mutant *APC* constructs (2 µg) were transfected into 293, SW480, and HCT116 cells using Lipofectamine (GIBCO/BRL, Gaithersburg). Protein was harvested 24 hours later and subjected to immunoblot analysis with APC monoclonal antibody FE9 (23). In HCT116 and 293 cells, exogenous WT APC comigrated with the endogenous APC. In SW480 cells, APC1309Δ comigrated with the endogenous mutant APC. In all other cases, the nonfunctional APC constructs (APC331Δ, APC 1309Δ, and APC1941Δ) produced as much or more protein than the CRT-functional forms of APC (APC WT and APC 2644Δ).

EXAMPLE 6

This example demonstrates that other components of the APC-regulatory pathway are affected in some cancer cells.

We evaluated CRT in two colorectal tumor cell lines (HCT116 and SW48) that express full-length APC (Fig. 6A). Both HCT116 and SW48 displayed constitutively active CRT and, in contrast to cell lines with truncated APC (DLD1 and SW480), this activity was not inhibited by exogenous WT APC (Fig. 5B, 6B). Other (noncolorectal cancer) cell lines expressing WT APC do not display constitutive CRT activity. These transfection results suggested that the constitutive CRT in HCT116 and SW48 might be due to an altered downstream component of the APC tumor suppressor pathway.

EXAMPLE 7

This example demonstrates a defect in the gene encoding β -catenin in some cancer cells, which affects CRT.

We evaluated the status of a likely candidate for a downstream component of the APC tumor suppressor pathway, β -catenin, in the same four lines. All four lines expressed similar amounts of apparently intact β -catenin, as assessed by immunoblots (Fig. 7A). However, sequence analysis revealed that both HCT116 and SW48 harbored mutations in the β -catenin gene (*CTNNB1*) (Fig. 7B). HCT116 had a 3-bp deletion that removed one AA (Ser-45), and SW48 had a C to A missense mutation that changed Ser-33 to Tyr. Analysis of paraffin-embedded archival tissue from the HCT116 patient confirmed the somatic nature of this mutation and its presence in the primary tumor prior to culture. Interestingly, both mutations affected serines that have been implicated in the down regulation of β -catenin through phosphorylation by the ZW3/GSK3 β kinase in *Xenopus* embryos (Fig. 7C) (27,28).

Genomic DNA was isolated from paraffin-embedded normal and tumor tissue from the patient from whom the HCT116 cell line was derived. A 95 bp PCR product encompassing the mutation was then amplified by PCR and directly sequenced using THERMOSEQUENASE (Amersham). The 3 bp deletion was observed in tumor but not in normal tissue.

To test the generality of this mutational mechanism, we evaluated five primary colorectal cancers in which sequencing of the entire coding region of *APC* revealed no mutations (25). Three of these five tumors were found to contain *CTNNB1* mutations (S45F, S45F, and T44A) that altered potential ZW3/GSK3 β phosphorylation sites (Fig. 7C). Each mutation appeared to affect only one of the two *CTNNB1* alleles and to be somatic.

Genomic DNA was isolated from frozen-sectioned colorectal cancers and a 1001 bp PCR product containing exon 3 of *CTNNB1* was then amplified by PCR and directly sequenced using ThermoSequenase

(Amersham). An ACC to GCC change at codon 41 (T41A) and a TCT to TTT at codon 45 (S45F) was observed in one and two tumors, respectively.

EXAMPLE 8

This example demonstrates dominant mutations of *CTNNB1* that render CRT insensitive to the effects of WT APC.

Because the β -catenin mutations were heterozygous, we hypothesized that the mutations might exert a dominant effect, rendering a fraction of cellular β -catenin insensitive to APC-mediated down regulation. To test this notion, we performed gel shift analyses with nuclear extracts from untransfected HCT116 cells. In contrast to noncolorectal cancer cell lines with intact *APC*, HCT116 cells contained a β -catenin/Tcf complex that gel-shifted an optimized Tcf-binding oligonucleotide, and this complex supershifted with anti- β -catenin (Fig. 8A). We also constructed β -catenin expression vectors and compared the biologic activity of the mutant β -catenin from HCT116 (β -Cat Δ 45) and SW48 (β -Cat S33Y) with that of their WT counterpart. For these experiments, we used the 293 kidney epithelial cell line as it is highly transfectable, exhibits low endogenous CRT, and contains a high level of endogenous APC (Fig. 6A). In the presence of endogenous APC, both mutant β -catenins were at least 6-fold more active than the WT protein and this activity was inhibited by dominant-negative hTcf-4 (Fig. 8B).

Together, these results indicate that disruption of APC-mediated regulation of CRT is critical for colorectal tumorigenesis. This is most commonly achieved by recessive inactivating mutations of both *APC* alleles but, as shown here, can also be achieved by dominant mutations of *CTNNB1* that render CRT insensitive to the effects of WT APC. Our results suggest that APC inhibition of CRT requires phosphorylation of β -catenin at multiple sites. These potential phosphorylation sites are consistent with the known specificity of ZW3/GSK3 β (29) a serine kinase that negatively regulates

5 β -catenin in *Xenopus* and *Drosophila* cells (27) and that interacts with APC and β -catenin in mammalian cells (23). These results also suggest a functional basis for the occasional *CTNNB1* mutations observed in other tumor types (30) and illustrate how a critical pathway in human disease can be illuminated by the discovery of mutations in different components of the pathway. The next step in understanding *APC* function will be the identification of the genes that are activated by hTcf-4/ β -catenin complexes and inhibited by WT APC. These genes are likely to be related to APC's ability to induce apoptosis in colorectal cancer cells (31).

EXAMPLE 9

Characterization of the Ad-Mini-ME recombinant adenovirus.

Understanding the structure-function relationships of APC has been hindered by the inability to readily restore APC tumor suppressor activity to human cells. To address this problem, we developed an adenovirus system that would allow the relatively facile evaluation of APC effects in a variety of cell lines. Three features of the adenovirus construction are of particular interest. First, to facilitate the actual construction of the adenoviral vectors, we used the AdEasy adenovirus system, which employs recombination in bacteria rather than in mammalian cells to generate recombinant adenovirus. Second, we included a GFP marker to allow easy identification of APC expressing cells. This eliminates problems related to differences in infectivity and allows the use of viral titers well below levels that result in virus-induced cytopathic effect (CPE). Avoiding such nonspecific CPE is especially important for assessing tumor suppressive effects. Finally, we generated a virus that expressed a fusion protein (GFP/cAPC) containing GFP fused to the central third of APC (Fig. 9A). The employment of a fusion protein ensured that expression of GFP was coupled with APC and allowed positive verification of cAPC expression. The growth suppressive effects of tumor suppressor genes impose a powerful negative selection force that can result in loss of expression of the tumor suppressor gene even in the presence of a positive selection marker.

The central portion of APC was chosen for the following experiments because it contains all of the known β -catenin and Axin/Conductin binding domains (Fig. 9A) and is sufficient for promoting β -catenin degradation. In keeping with precedent, the clone expressing this smaller version of APC Minus its amino- and carboxyl-terminal Ends was designated Ad-Mini-ME. (37) Infection of 293 and SW480 cells with Ad-Mini-ME resulted in the

production of a fusion protein of the expected size (150 kD fusion) as judged by Western blot analysis with an anti-GFP antibody (**Fig. 9B**). Fluorescence microscopy revealed that the GFP/cAPC fusion protein was diffusely present in the cytoplasm (**Fig. 9C**).

5

Ad-Mini-ME inhibits Tcf4/ β -catenin mediated transactivation.

One of the best-characterized functions of APC is its ability to inhibit CRT. To investigate the effects of GFP/cAPC on this function, we generated stable DLD1 cell lines with either an integrated Tcf-4 responsive luciferase reporter (DOT cells) or a reporter driven by the SV40 promoter (Dluc cells). Infection of DOT cells with Ad-Mini-ME markedly suppressed luciferase activity, whereas a virus expressing GFP alone (Ad-GFP) had no effect (**Fig 10A**). This inhibition appeared to be specific as infection of Dluc cells with Ad-Mini-ME had no effect on luciferase activity (**Fig. 10B**). To determine whether the Ad-Mini-ME suppression could be extended to endogenous targets of CRT, we evaluated the expression of c-MYC and cyclin D1, two recently described targets of the APC/ β -catenin/Tcf-4 pathway. Expression of c-MYC and cyclin D1 was examined in three human colorectal cancer cell lines. In DLD1 and SW480 CRT was constitutively activated because of APC mutations, while in HCT116 CRT activation was due to a β -catenin mutation.

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Infection of SW480 and DLD1 cells with Ad-Mini-ME resulted in a marked reduction of c-MYC protein levels, and a minor reduction of cyclin D1 levels (**Fig. 11**). Ad-GFP infection had no inhibitory effect on either c-MYC or cyclin D1 protein levels in these cells. As expected, HCT116 cells, which possess a stabilizing β -catenin mutation, were relatively resistant to the effects of Ad-Mini-ME (**Fig. 11**).

25

The subcellular localization of β -catenin was dramatically altered by Ad-Mini-ME infection. Whereas mock-infected or Ad-GFP-infected DLD1 cells displayed predominantly nuclear β -catenin staining, cells infected with

Ad-Mini-ME showed cytoplasmic and membrane β -catenin staining with minimal nuclear staining (**Fig. 12A**). A similar alteration in the subcellular distribution of β -catenin was observed in SW480 cells (**Fig. 12B**). The selective depletion of nuclear β -catenin by Ad-Mini-ME is consistent with the model for APC action proposed previously.

Ad-Mini-ME suppresses cell growth and colony formation in colorectal cell lines.

In both DLD1 and SW480, Ad-Mini-ME infection resulted in a clear growth inhibition by day 2 (**Fig. 13A**). This growth inhibition was persistent, with Ad-Mini-ME infected DLD1 and SW480 cells failing to reach confluence even after 2 weeks of culture, long after mock- and Ad-GFP infected cells became superconfluent. Consistent with the effects of Ad-Mini-ME on CRT in HCT116 cells, the growth of Ad-Mini-ME infected HCT116 cells was only partially inhibited (**Fig. 13A**).

We next examined the effects of Ad-Mini-ME mediated CRT inhibition on colony formation in collagen gels using a series of eight colorectal cancer cell lines. The cells were flow sorted to select virally-infected cells, as described in Materials and Methods. Infection with Ad-Mini-ME resulted in a marked suppression of colony formation in SW480, DLD1, LoVo, HT29, SW837, and SW1417 cells, all of which possess mutated APC (**Fig. 13B**). Colony numbers were reduced by at least 96% in each of these lines compared with cells mock-infected or infected with Ad-GFP virus. Examination under phase-contrast microscopy revealed that most of the Ad-Mini-ME infected cells were pyknotic. A small number of growth-arrested single cells remained in the gels, and these continued to express APC as judged by fluorescence. In the few colonies that did form after Ad-Mini-ME infection, APC did not appear to be expressed. In contrast, cells with intact APC but mutated

β -catenin were able to form a significant number of colonies following Ad-Mini-ME infection (HCT116 and SW48 in Fig. 13B).

Ad-Mini-ME induces apoptosis in colorectal cell lines.

Ad-Mini-ME-expressing cells revealed a gradual loss of the G1 peak and an accumulation of cells in the S and G₂ phases of the cell cycle. Five days after infection, all six colorectal cell lines with *APC* mutations demonstrated significantly increased apoptosis following Ad-Mini-ME infection (Fig. 14). In line with the effects of Ad-Mini-ME on CRT and growth, the mutant β -catenin containing cell lines, HCT116 and SW48, exhibited little increase in apoptosis in response to Ad-Mini-ME infection. Time course studies revealed that the first morphological signs of apoptosis were not evident in DLD1 and SW480 cells until 72 hours after plating. The induction of apoptosis was confirmed by Annexin V, which has been shown to bind to phosphatidylserine exposed on the outer leaflet of apoptotic cell membranes. The proportion of DLD1 and SW480 cells staining with Alexa568-labeled annexin V was in good agreement with the fraction of cells displaying morphological signs of apoptosis (>90% of annexin V-labeled cells displayed apoptotic nuclei).

Tumor growth in nude mice.

To investigate the effects of Ad-Mini-ME on colorectal cancer cells in vivo, Ad-GFP and Ad-Mini-ME-infected DLD1 cells were selected by flow sorting and injected subcutaneously into nude mice. Mice sacrificed three days after injection showed a large number of apoptotic tumor cells at the injection site (Fig. 15, H&E). Such changes were not evident after injection of mock-infected or Ad-GFP-infected cells. Apoptosis was further evaluated by immunoreactivity with the M30 antibody, which recognizes a caspase-cleaved epitope of cytokeratin 18. Ad-Mini-ME infected cells were diffusely positive for the M30 antibody, while only a small fraction of

Ad-GFP-infected cells stained with this antibody (Fig. 15, M30). Immunoperoxidase staining with an antibody specific for the proliferation marker Ki-67 showed a significant decrease in nuclear labeling of Ad-Mini-ME infected tumors, with only 10% of cells positive (Fig. 15, Ki-67). In contrast, over 90% of Ad-GFP-infected cells were Ki-67 positive. Tumors derived from Ad-GFP infected cells continued to grow in the nude mouse, eventually forming tumors larger than 1 cm in diameter, while there was no visible tumor formation after injection of Ad-Mini-ME infected cells after three weeks.

Materials and Methods

Cell Culture, Medium, and Reagents. 293 cells were purchased from Microbix Biosystems (Toronto, Canada) and 911 cells were kindly provided by Alex J. Van der Eb of the University of Leiden. Both lines were maintained in DMEM growth medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml of penicillin and 100 µg/ml of streptomycin. The human colon carcinoma cell lines, SW480, DLD1, HCT116, LoVo, SW48, HT29, SW837, and SW1417 were obtained from the American Type Culture Collection and cultivated in McCoy's 5A media (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml of penicillin and 100 µg/ml of streptomycin. The DOT and Dluc cell lines were derived from DLD1 cells as described below. Both cell lines were maintained in McCoy's 5A media (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone) and 0.4 mg/ml Hygromycin B (Calbiochem).

Generation of Recombinant Adenovirus Expressing Central Third of APC (Ad-Mini-ME). The recombinant adenovirus, Ad-Mini-ME, which

carried the central third of APC gene, was generated using a modified system as previously described. The central third of APC containing amino acids 958-2075 (nucleotides 2890-6240) was isolated from pCMV-APC by Bgl II digestion. This fragment was subcloned into the pEGFP- C1 (Clontech, Palo Alto, CA). The cassette containing the EGFP-tagged central third of APC gene (cAPC) was further subcloned into the shuttle vector (pShuttle) using Apal I and Mlu I digestion. Recombinant adenoviral plasmid was generated by homologous recombination in *E. coli* (BJ5183). BJ5183 cells were transformed using electroporation with pAdEasy-1 and pShuttle/EGFP-cAPC linearized with PmeI. Successful recombinants were identified by restriction endonuclease mapping. The recombinant EGFP-cAPC virus (Ad-Mini-ME) was produced in the 911 and 293 adenovirus packaging lines and the viral particles were purified by CsCl banding. The control virus (Ad-EGFP) with EGFP alone was also prepared and purified side by side. Viral titer was determined by a modified CPE³ endpoint assay. A series of Ad-GFP infections was performed on HCT116 cells to determine the optimal multiplicity of infection (MOI) to avoid adenovirus-associated CPE. Typically, viral CPE could be observed at an MOI of above 100, which resulted in more than 80% of cells becoming fluorescent 18 hours after Ad-GFP infection. To avoid any cytopathic effects of viruses, we infected cells with a minimal MOI, generating fluorescence in 20-30% of the cells (MOI=5-11), then flow-sorted infected cells to obtain homogeneous populations.

Viral Infection and Cell Sorting. Viral stocks were predialyzed using 1% agarose in microcentrifuge tube. Three million cells were infected with either Ad-Mini-ME or Ad-GFP in a 75 cm² flask. After 18 hours of incubation at 37°C, cells were washed, trypsinized and subjected to fluorescence activated cell sorting. Cells with green fluorescence were

collected for experiments or were replated in culture flasks immediately after sorting.

Reporter Assay. DOT and Dluc cells were generated from DLD1 cells by cotransfection of pTK-hygro (Clontech) and a Tcf-4 responsive luciferase plasmid (pGL3-OT, Yu, J. unpublished data) or a constitutive luciferase plasmid (pGL3-control, Promega, Madison, WI), respectively. Clones were isolated and the sensitivity to CRT determined using a dominant negative Tcf-4 adenovirus (Yu, J. unpublished data). Luciferase reporter activity in the DOT clone was constitutively high as expected for a CRT responsive reporter in a colorectal cancer cell line with mutated APC. This constitutive activity was inhibited by dominant negative Tcf-4. In contrast, the luciferase activity in the Dluc clone was unaffected by dominant negative Tcf-4 as expected for expression driven by the SV40 promoter. To assess the effects of cAPC on CRT, DOT and Dluc cells were infected with Ad-GFP and Ad-Mini-ME. Eighteen hours after viral infection, equal numbers of GFP positive cells were pelleted, lysed, and collected for luciferase assays using Luciferase Assay Reagents (Promega).

Western Blot Analysis. Whole cell lysates were prepared in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 0.5% β -mercaptoethanol. Equal amounts of total protein from each lysate were loaded and separated on 4-12% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes. Western blots were developed by chemiluminescence (NEN Life Science, Boston, MA), detected by Kodak Image Station 440CF, and analyzed by 1D Image Analysis software (NEN Life Science). Primary antibodies included anti-GFP polyclonal antibody from Clontech (Palo Alto, CA), anti-c-MYC monoclonal antibody (9E10)

from Santa Cruz (Santa Cruz, CA), anti-cyclin D1 monoclonal antibody (A-12) from Santa Cruz, and anti- α -tubulin monoclonal antibody (TU-02) from Santa Cruz. Secondary peroxidase-conjugated antibodies were goat anti-mouse IgG and goat anti-rabbit IgG from Pierce (Rockford, IL).

5 **Immunofluorescence Staining.** Cells were infected with Ad-GFP or Ad-Mini-ME for 18 hours and sorted. Fluorescent cells were cultured on 8-well chamber CultureSlides (Becton Dickinson, Bedford, MA). After 8 hours, cells were fixed in 3% paraformaldehyde in PBS at room temperature for 8 min, then permeabilized with 0.3% NP-40 in PBS for
10 another 8 min. After washing in PBS, the cells were incubated with primary mouse anti- β -catenin monoclonal antibody (1 μ g/ml; Transduction Laboratories, Lexington, KY) at 4°C overnight. After washing, cells were incubated with biotinylated goat anti-mouse IgG (Pierce, Rockford, IL) at room temperature for 1 hour. The immunoreactivity was revealed using
15 Alexa568-conjugated streptavidin (Molecular Probes, Eugene, OR) and cells were counterstained with 10 μ g/ml Hoechst 33258. The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD).

20 **Cell Growth and Colony Formation Assay.** 10^5 cells were plated in one well of a 24 well-plate. Cells were counted using a hemocytometer after trypsinization on days 1, 2, 3 and 5. For colony formation assays, each well of 24-well plates was precoated with 100 μ l of collagen gel containing 50% type I collagen (Collaborative Biomedical Science, Bedford, MA), 40% culture medium, and 0.75% NaHCO_3 (Halttunen). 160 μ l of collagen
25 gel-cell suspension containing 10,000 cells, 45% type I collagen, 40% culture medium, and 0.075% NaHCO_3 was added to the wells. After solidification, each well was covered with 1 ml of culture medium and the

plates were incubated at 37°C. Twelve days after seeding, cells were stained with 0.05% crystal violet (Sigma, St. Louis, MO) containing 10% buffered formalin (Sigma).

Hoechst Staining and Annexin V Staining for Apoptosis Detection.

5 Both attached and floating cells were harvested for staining. For Hoechst staining, 3×10^5 cells were resuspended in 50 μ l PBS and 350 μ l of staining solution containing 0.6% Nonidet P-40, 3% paraformaldehyde, and 10 μ g/ml Hoechst 33258. For annexin V staining, 10^5 cells were suspended in 100 μ l annexin-binding buffer containing 10mM HEPES, 140 mM NaCl
10 and 2.5 mM CaCl_2 . 5 μ l of Alexa568-conjugated annexin V (Molecular Probes) was added and incubated at room temperature for 15 min, at which point an additional 400 μ l of annexin-binding buffer was added to each sample. Apoptotic cells were defined as those cells containing condensed and/or fragmented nuclei after Hoechst staining or were fluorescent after
15 annexin V staining. At least 500 cells were counted and the results were expressed as the percentage of apoptotic cells in each sample.

Experimental Animals and Immunoperoxidase. Eight pathogen-free female athymic nude mice (Harlan, Indianapolis, IN), 6-8 weeks old were injected with 3×10^6 DLD1 cells infected either with Ad-GFP or
20 Ad-Mini-ME in the subcutaneous tissues adjacent to the lower spine. Before injection, these cells were sorted and green fluorescent cells were collected and cultured for 24 hours. Three days after injection, two of the mice from each group were sacrificed and the tumors were formalin-fixed, paraffin-embedded, and processed for routine histological examination.
25 Tissue sections of 5 micron thickness were deparaffinized and incubated with a mouse anti-Ki-67 antibody (AMAC Inc., Westbrook, ME) or with a mouse antibody specific for a caspase-cleaved epitope of cytokeratin 18

(M30, Boehringer Mannheim, Mannheim, Germany). Cells were incubated with antibodies at 4°C overnight, then washed and developed with an avidin-biotin complex peroxidase method (Biogenex, San Ramon, CA). Immunoreactivity was detected using the 3,3'-diaminobenzidine chromagen and counterstained with 0.1% hematoxylin (Sigma, St Louis, MO).

DISCUSSION

While the ability of APC to suppress intestinal tumorigenesis has been known for several years, the sequences required for this inhibition have not been well defined. In this study, we define a minimal portion of APC that is sufficient for its growth suppressive effects. Our results suggest that expression of the central third of APC is sufficient to inhibit cellular proliferation and induce apoptosis in colorectal cancer cells.

The biological effects of the central third of APC are likely related to abrogation of the APC/ β -catenin/Tcf-4 signaling pathway. This conclusion is based on the fact that Ad-Mini-ME inhibits β -catenin nuclear translocation (Fig. 12), suppresses β -catenin/Tcf-4 mediated transcription in reporter assays (Fig. 10), and downregulates the expression of targets of the APC/ β -catenin/Tcf-4 pathway (Fig. 11). Cellular proliferation and colony formation are dramatically suppressed by Ad-Mini-ME in cell lines which contain mutations in the *APC* gene, but are only partially inhibited in lines containing mutations of β -catenin that render it resistant to APC degradation.

At the cellular level, expression of the central third of APC eventually results in apoptosis of colorectal cancer cells containing APC mutations. This observation is consistent with those in a previous report, demonstrating apoptosis 60 hours after induction of full length APC expression. In both cases, the delay in appearance of apoptotic cells

suggests that APC-induced apoptosis may not be a direct result of suppression of CRT.

While the β -catenin binding domain in the central third of APC is sufficient for growth suppression by APC, it may not recapitulate all of the functions of this gene. For example, the carboxyl-terminal third of APC can associate with the human homolog of the *Drosophila* tumor suppressor gene discs large (hDLG) and EB-1. The latter has recently been implicated in the spindle checkpoint. In addition, a carboxyl-terminal fragment of APC has been shown to induce assembly and bundling of microtubules in vitro and has a role in directed cell migration. Like other canonical tumor suppressor genes, it is likely that APC functions at several levels to regulate cell growth and suppress neoplastic transformation. However, the finding that the middle third of APC is sufficient to inhibit tumor cell growth focuses further attention on the APC/ β -catenin interaction. Future experiments to understand the upstream regulators and downstream transducers of this interaction should shed further light on tumorigenesis associated with defects in the APC pathway.

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CLAIMS

1. A fusion protein which comprises an enzyme covalently linked to a portion of APC which comprises its β -catenin binding domain.
2. The fusion protein of claim 1 wherein the β -catenin binding domain consists of at least amino acids 958 to 2075.
3. The fusion protein of claim 1 wherein the enzyme is GFP.
4. An isolated and purified polynucleotide which encodes the fusion protein of claim 1.
5. An isolated and purified polynucleotide which encodes the fusion protein of claim 2.
6. An isolated and purified polypeptide comprising a portion of APC which comprises its β -catenin binding domain.
7. The isolated and purified polypeptide of claim 6 wherein the binding domain consists of at least amino acids 958 to 2075.
8. An isolated and purified polynucleotide which encodes the polypeptide of claim 6.
9. An isolated and purified polynucleotide which encodes the polypeptide of claim 7.
10. A method for inhibiting the growth of a tumor cell comprising: administering to a tumor cell a polynucleotide according to claim 8, whereby growth of the tumor cell is inhibited.
11. The method of claim 10 wherein the tumor cell is in vitro.
12. The method of claim 10 wherein the tumor cell is in a human body.
13. The method of claim 10 wherein the polynucleotide is part of an adenovirus.
14. A method for inducing apoptosis of a tumor cell comprising: administering to a tumor cell a polynucleotide according to claim 8, whereby apoptosis of tumor cells is induced.
15. The method of claim 14 wherein the tumor cell is in vitro.

16. The method of claim 14 wherein the tumor cell is in a human body.
17. The method of claim 14 wherein the polynucleotide is part of an adenovirus.
- 5 18. An adenovirus vector which comprises the polynucleotide of claim 8.
19. An adenovirus vector which comprises the polynucleotide of claim 9.
20. The adenovirus vector of claim 19 which is Ad-mini-ME.
- 10 21. The fusion protein of claim 1 which lacks at last one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.
- 15 22. The polynucleotide of claim 4 wherein the fusion protein lacks at least one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.
- 20 23. The isolated and purified polypeptide of claim 6 which lacks at least one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.
- 25 24. The isolated and purified polynucleotide of claim 8 wherein the polypeptide lacks at lest one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.
25. A method for treating a patient with colorectal cancer or other cancer associated with FAP, the method comprising the step of:

administering to the patient a nucleotide sequence comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

26. A method for treating a patient with colorectal cancer or other cancer associated with FAP, the method comprising the step of:
- 5 administering to the patient a polypeptide comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

FIG. 1A

hTcf-4E 1

hTcf-1E 1

[illegible]

34

41

[illegible]

72

11

S F R D K S R E S L E E A A K R Q D G G L F K G P P Y P G Y P F I M I P D L T S

114

116

P Y L P N G S V S P T A R T Y L Q M K W P L L D V Q A G S L Q S R Q A L K D A R
 G M Y K E T V Y S A F N L L M H Y P P P S G A G Q H P Q P

154

145

P	S	P	A	H	I	V	S	N	K	V	P	V	Q	H	P	H	H	V	H	P	L	T	P	L	I	T	Y	S	N	E	H	F	T	P	G	N	P
							:																														
P	P		P	L	H		K	A	N				Q	P	P	H	G	V	P				Q	L	S	L	Y	E	H	F	N	S	P	H	P		

FIG. 1C

hTcf-4E 430 P P I T D L S A P K K C R A R F G L D Q Q N N W C G P C R R K K K C V R Y I Q G
 | | : | | | | | | | | | | | | : | | : |
 hTcf-1E 389 T T D P G S P K K C R A R F G L N Q Q T D W C G P C R R K K K C I R Y L P G

470 E G S C L S P P S S D G S L L D S P P P S P N L L G S P P R D A K S Q T E Q T Q
 | | | | | | | | | | | | | | | |
 427 E G R C P S P V P S D D S A L G C P G S P A P Q D S P S Y H L L P R F P T E

510 P L S L S L K P D P L A H L S M M P P P P A L L L A E A T H K A S A L C P
 | : | | | | | : | | : |
 465 L L T S P A E P A P T S P G L S T A L S L P T P G P P Q A P R S T L Q S T Q V Q

547 N G A L D L P P A A L Q P A A P S S S I A Q P S T S W L H S S S L A G T Q P Q 3/23
 :
 505 Q Q E S Q R Q V A *

587 P L S L V T K S L E *

hTcf-4B 390 L Y P G W S A R D N Y G K K K K R R K R D K Q P G E T N G E K K S A F A T Y K V K
 | | | | | | | | | | | | : | | : | | |
 hTcf-1B 364 L Y P G W S A R D N Y G K K K R R S R E K H Q E S T T G G K R N A F G T Y P E K

430 A A A S A H P L Q M E A Y *
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 404 A A A P A P F L P M T V L *

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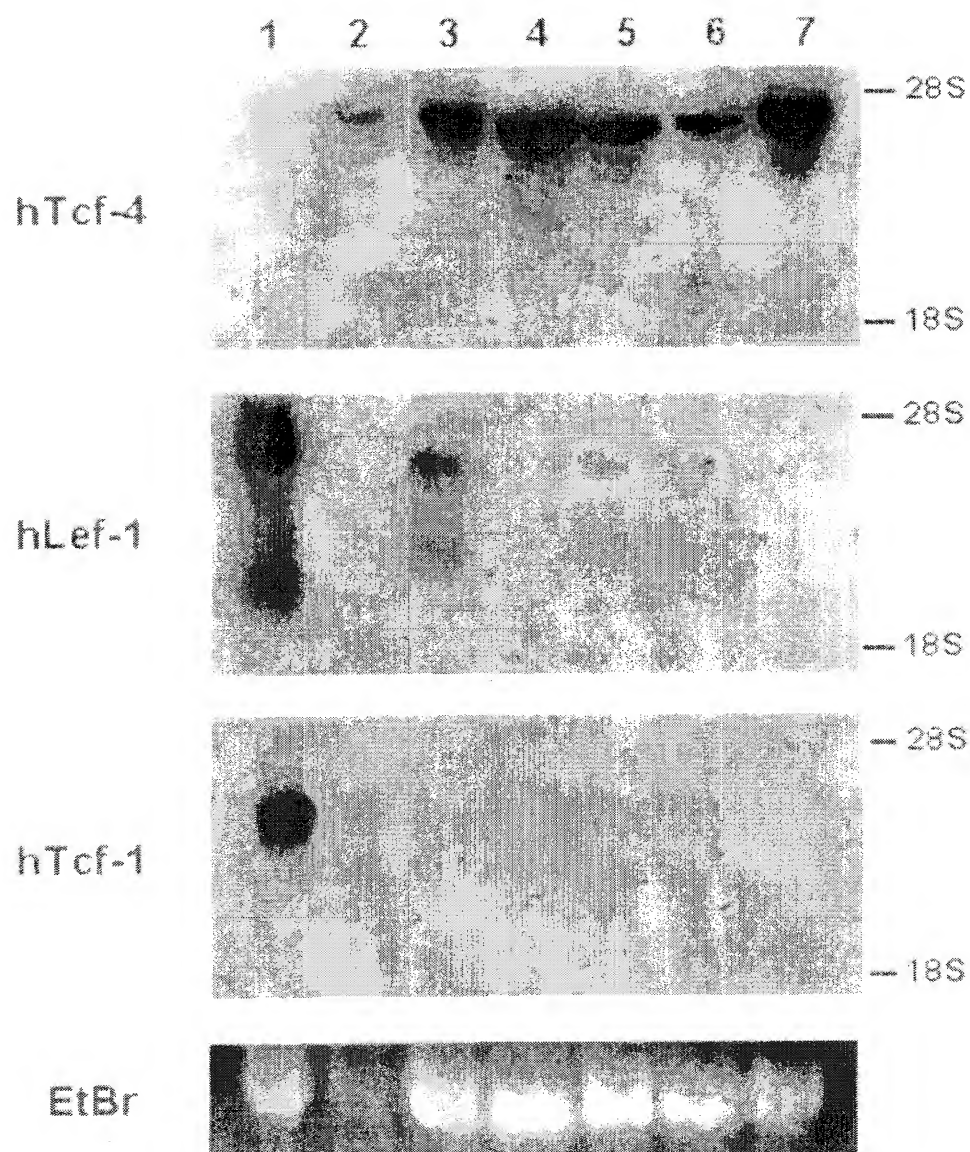
FIG. 2A

FIG. 2B

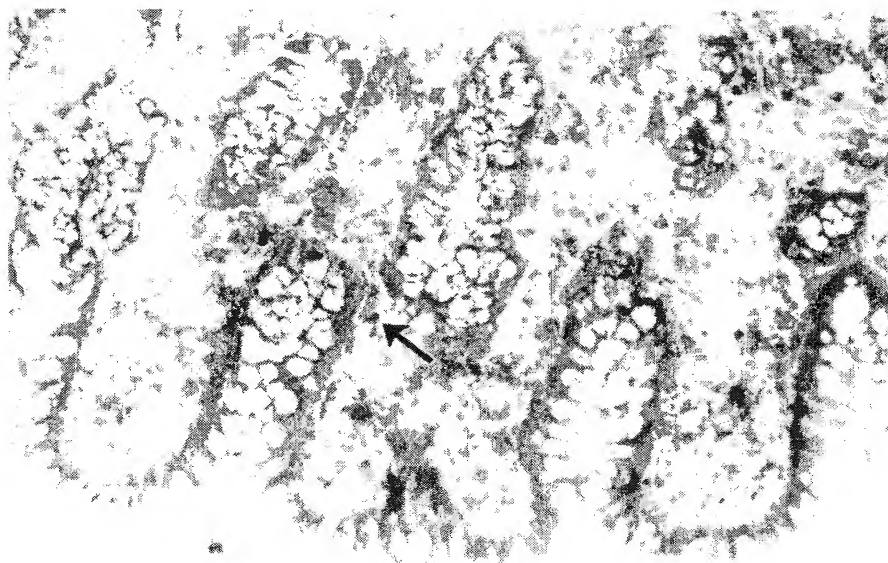
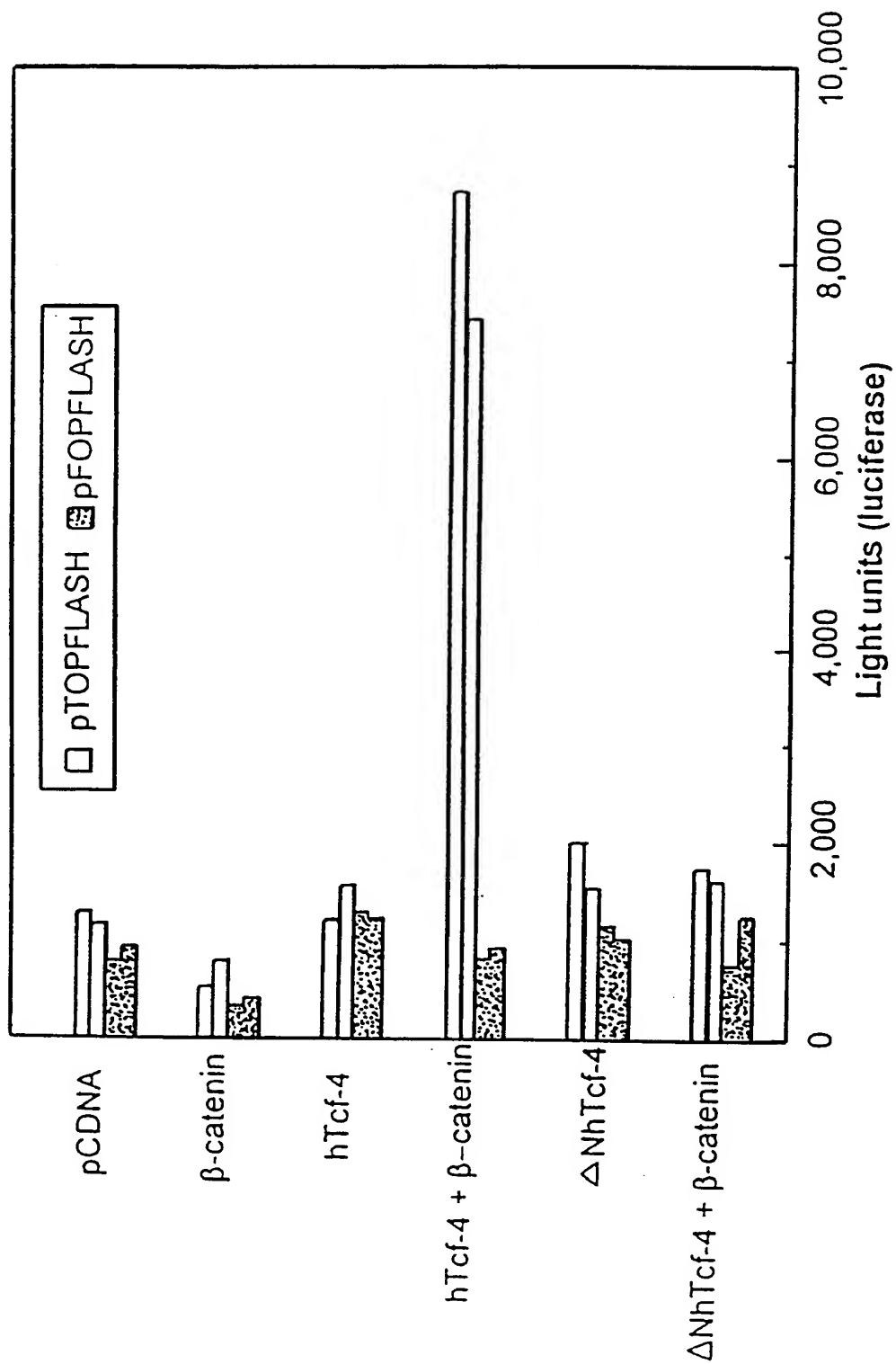


FIG. 2C



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FIG. 3A



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FIG. 3C

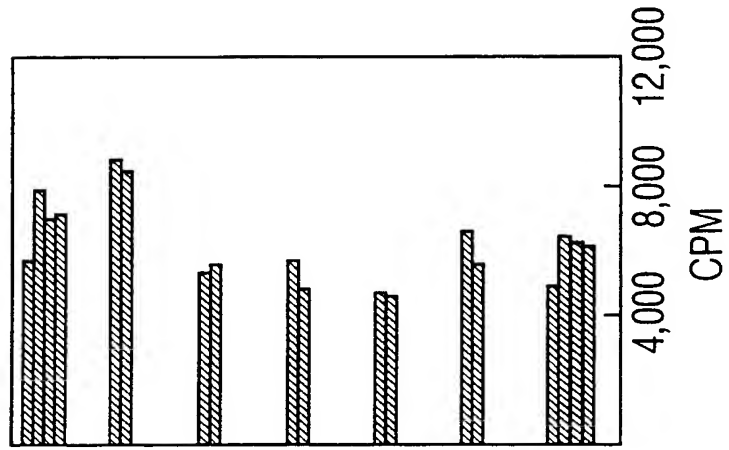
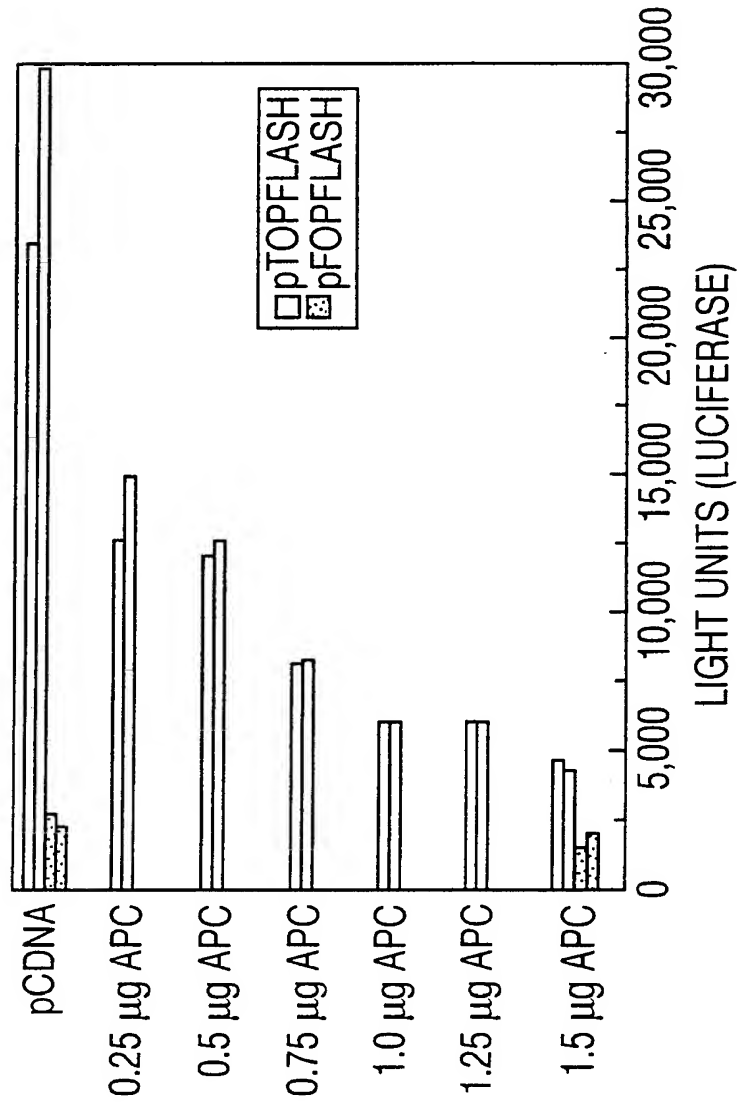


FIG. 3B



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FIG. 4

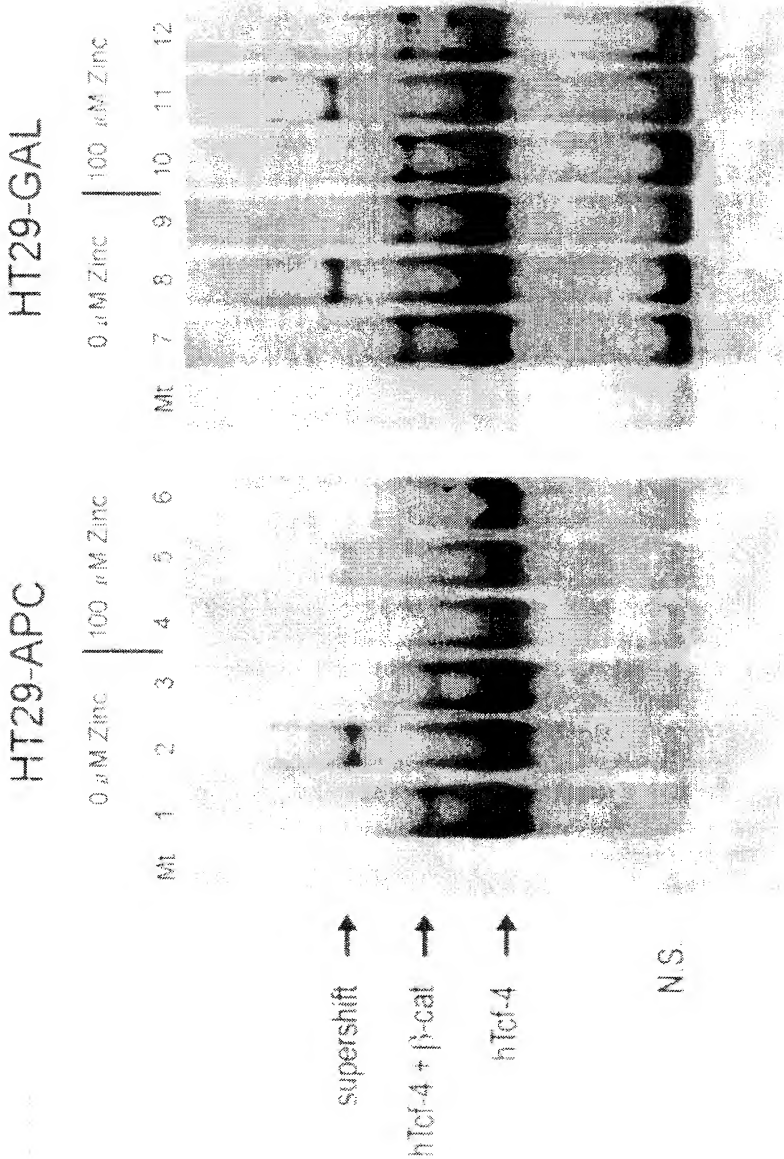
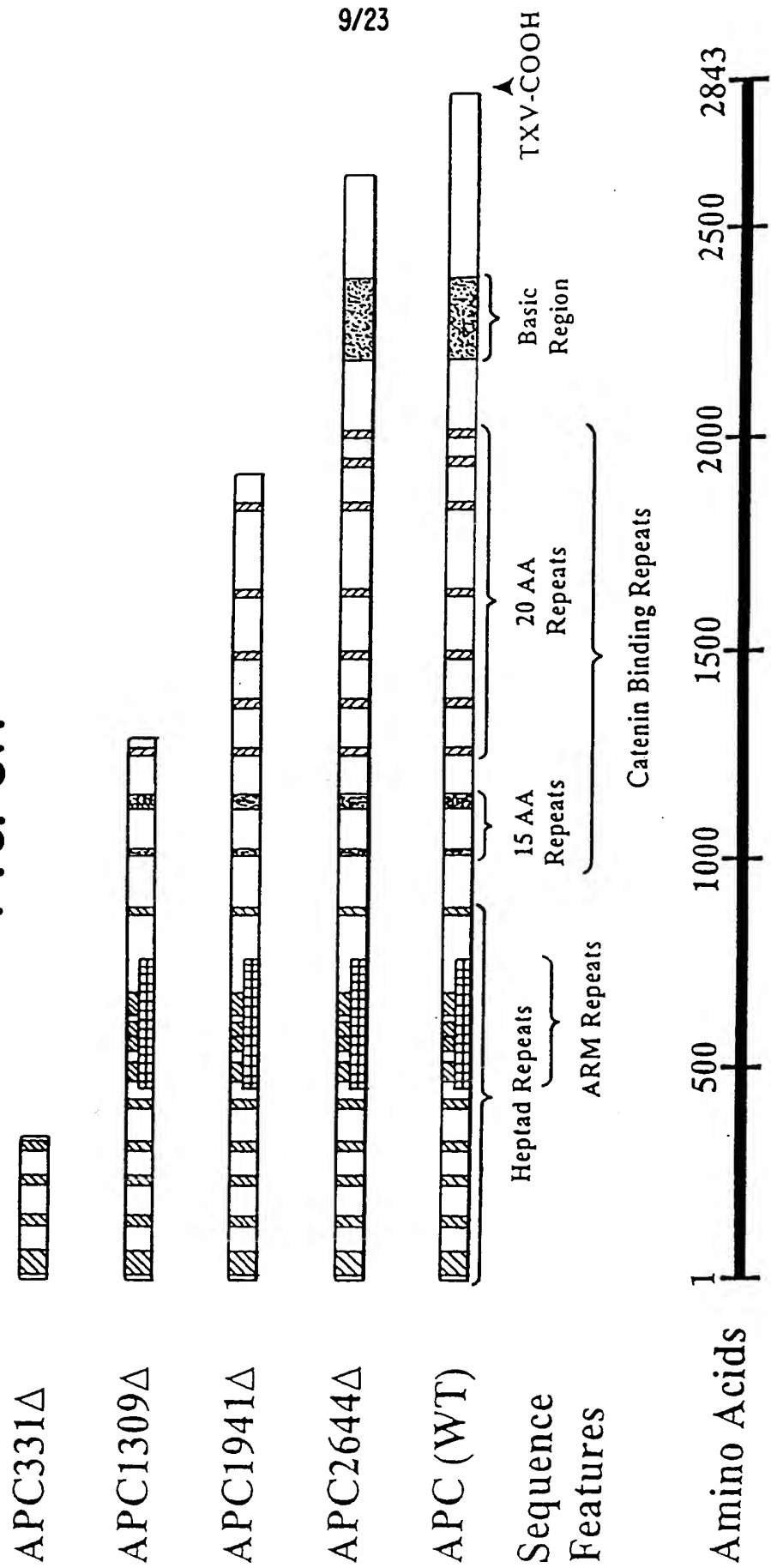


FIG. 5A



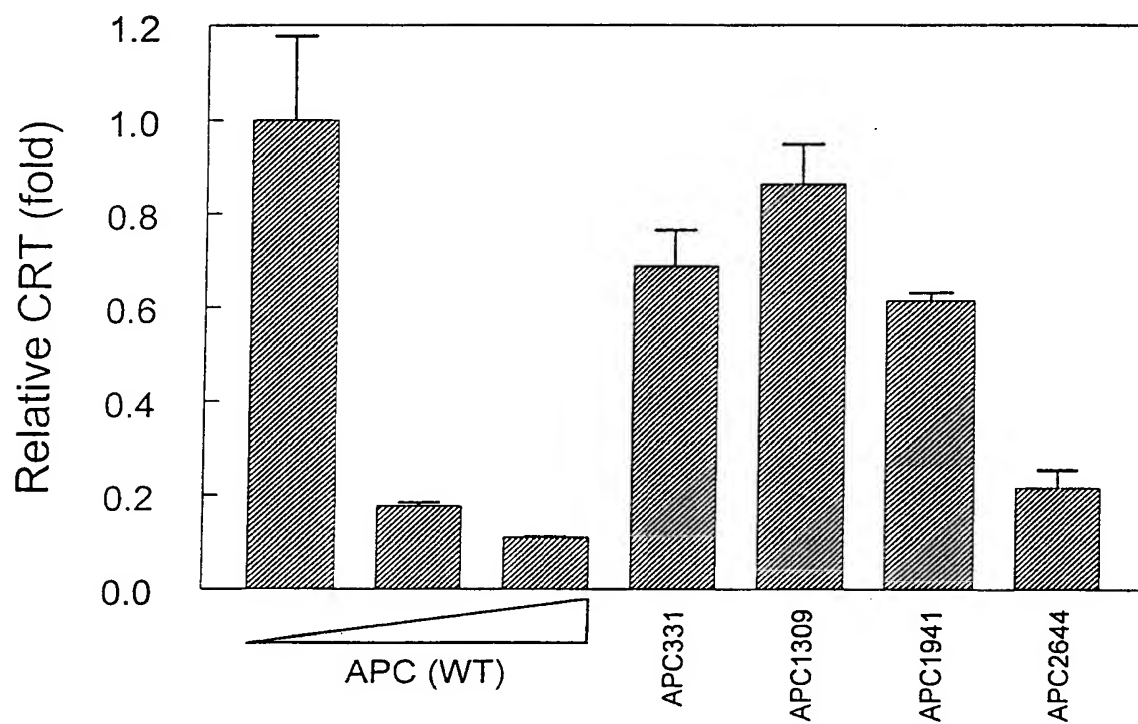
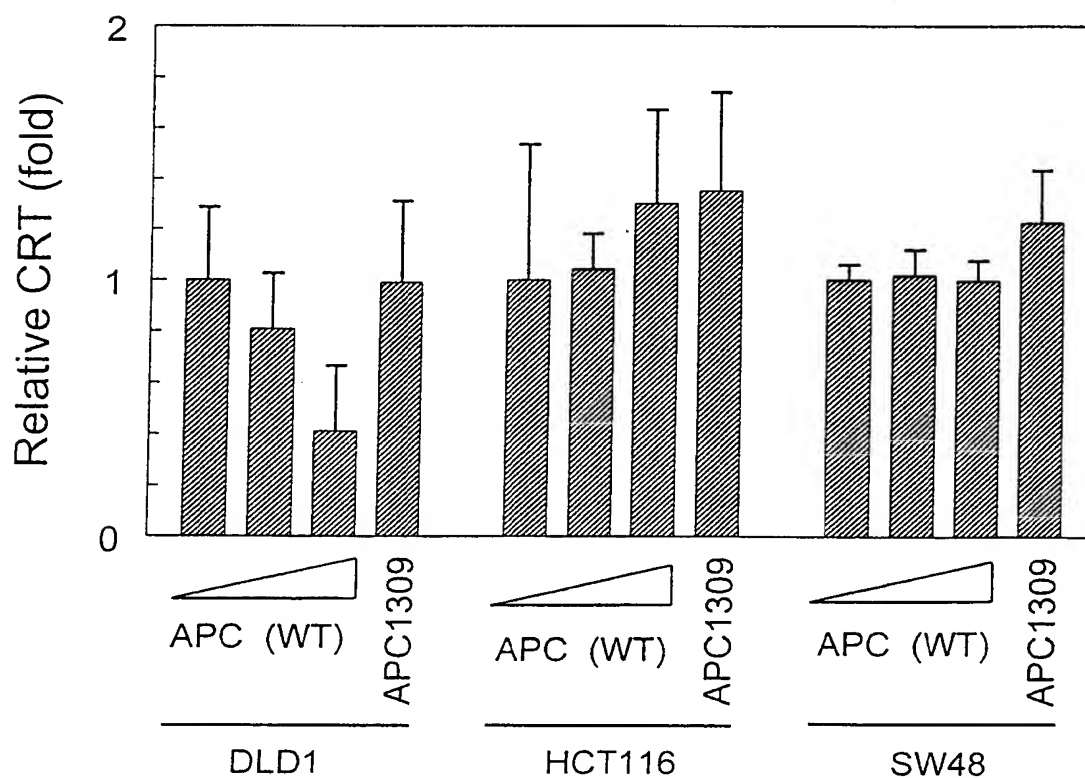
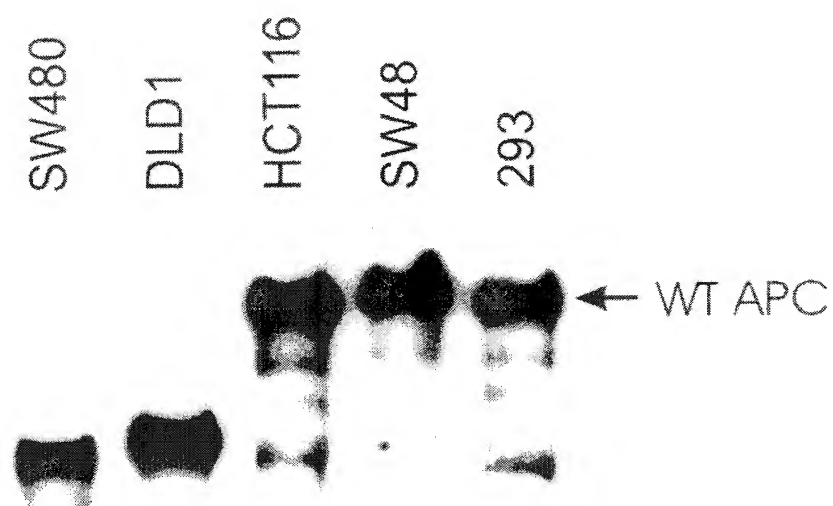
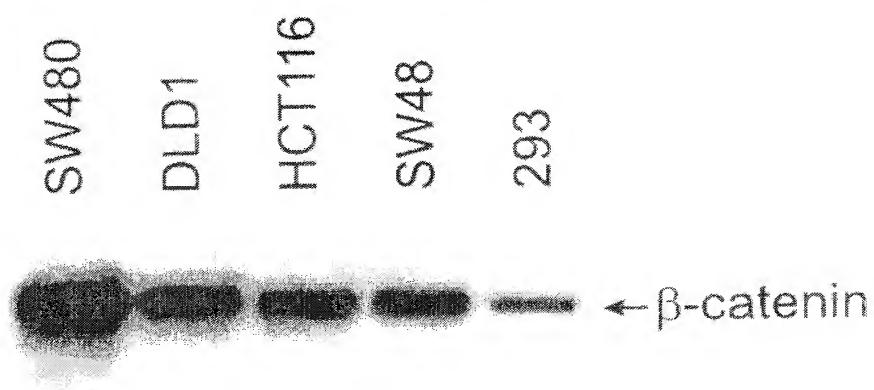
10/23
FIG. 5B**FIG. 6B**

FIG. 6A**FIG. 7A**

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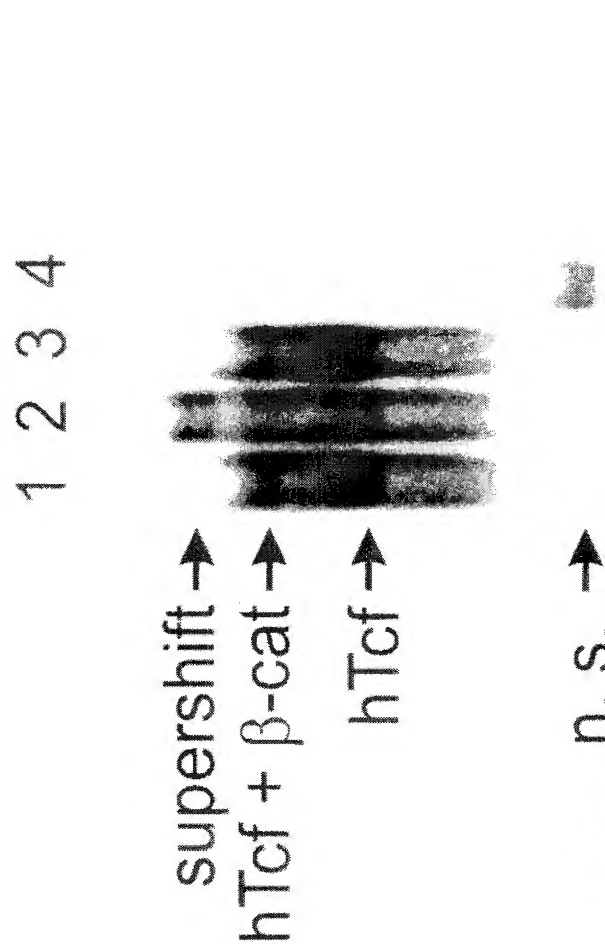


FIG. 7B

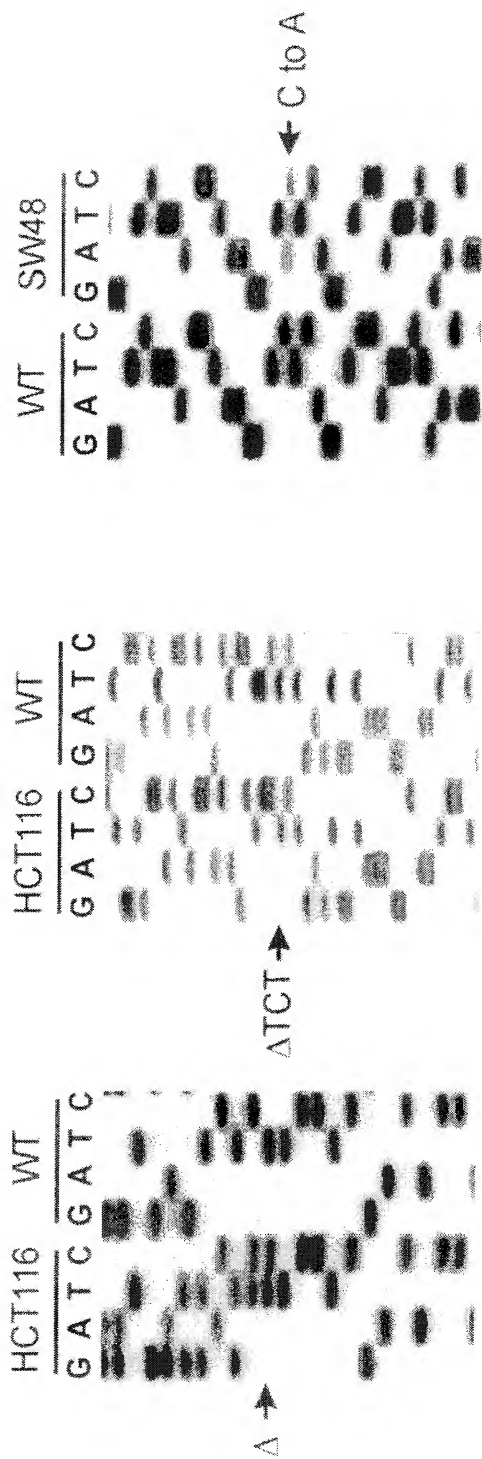


FIG. 7C

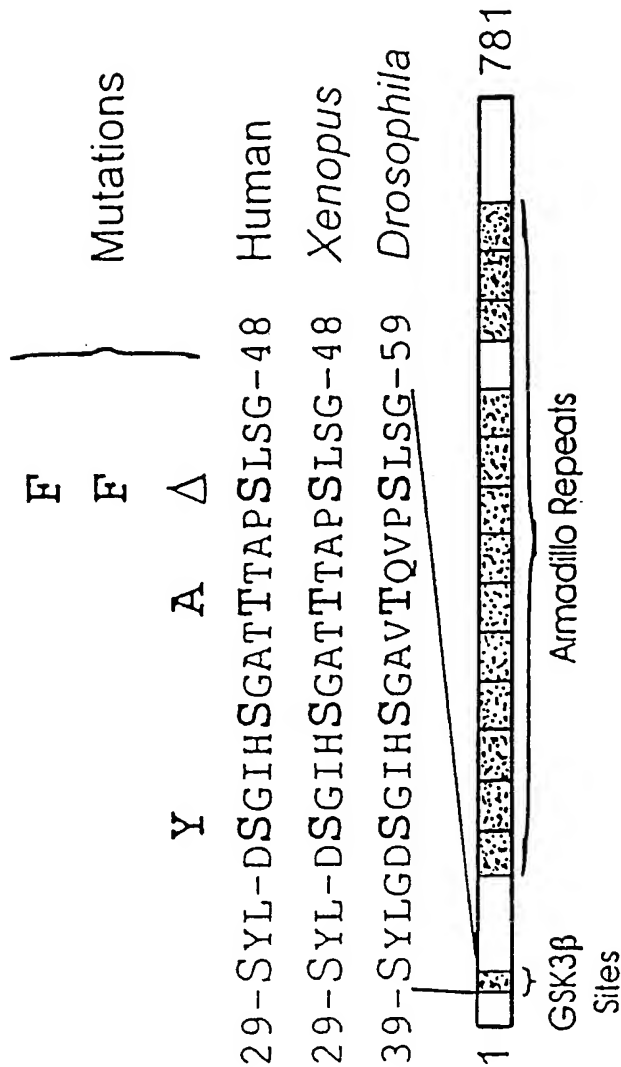


FIG. 8B

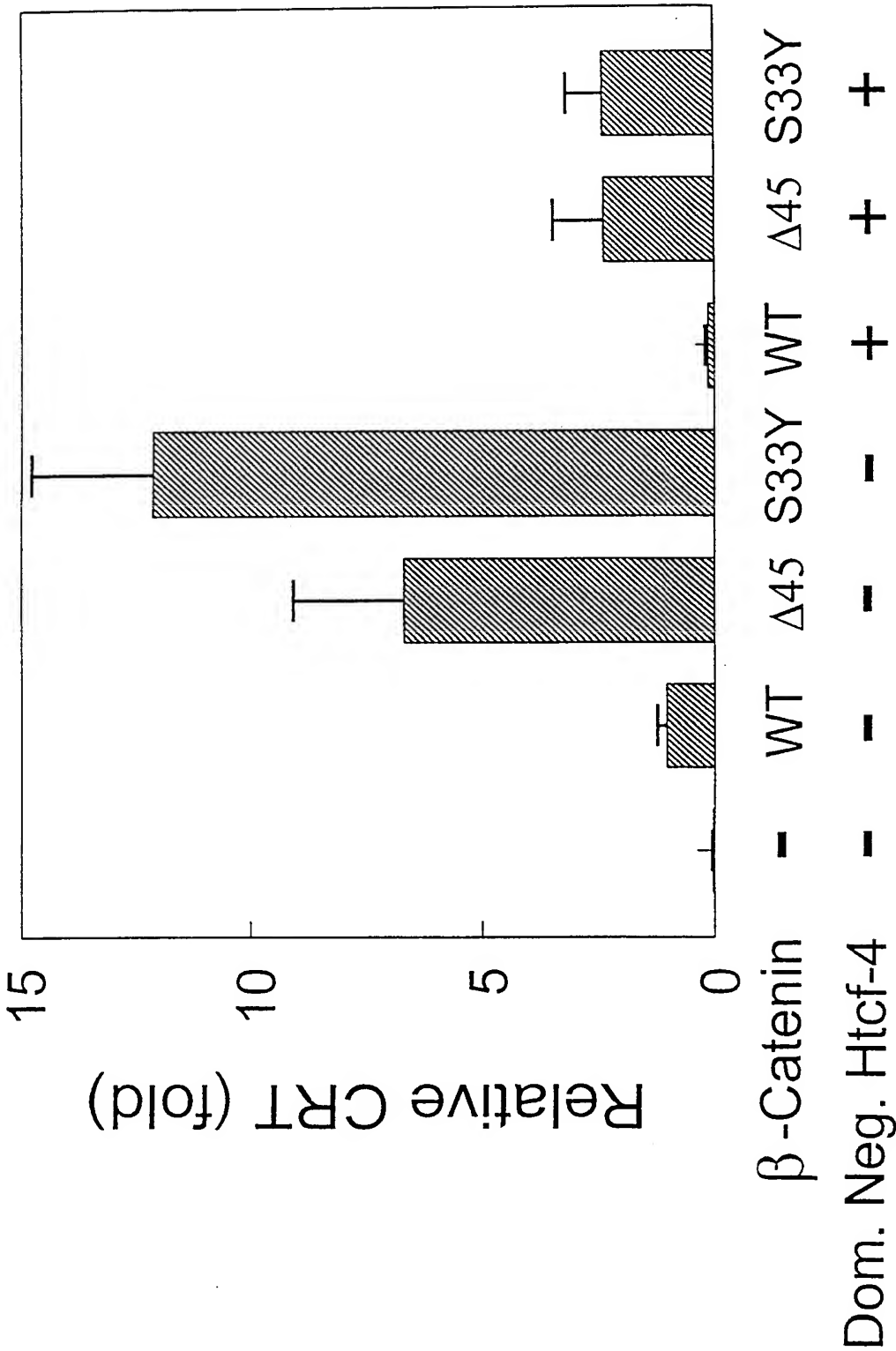
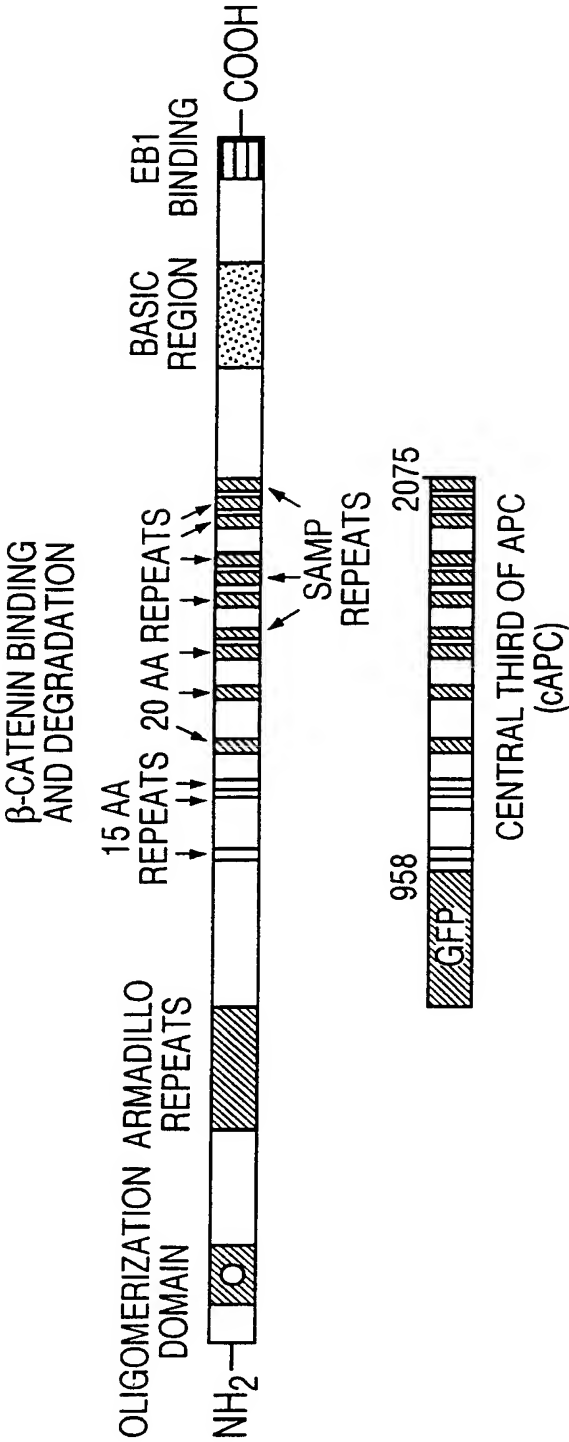
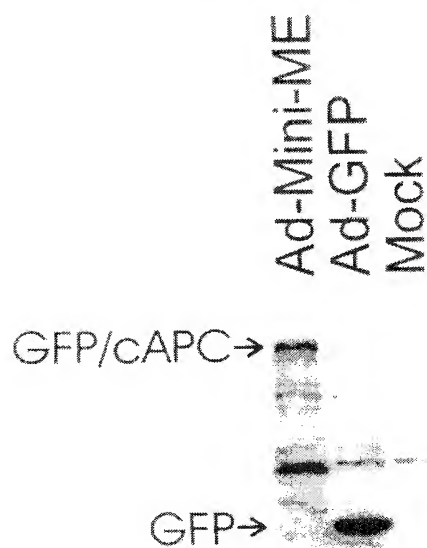
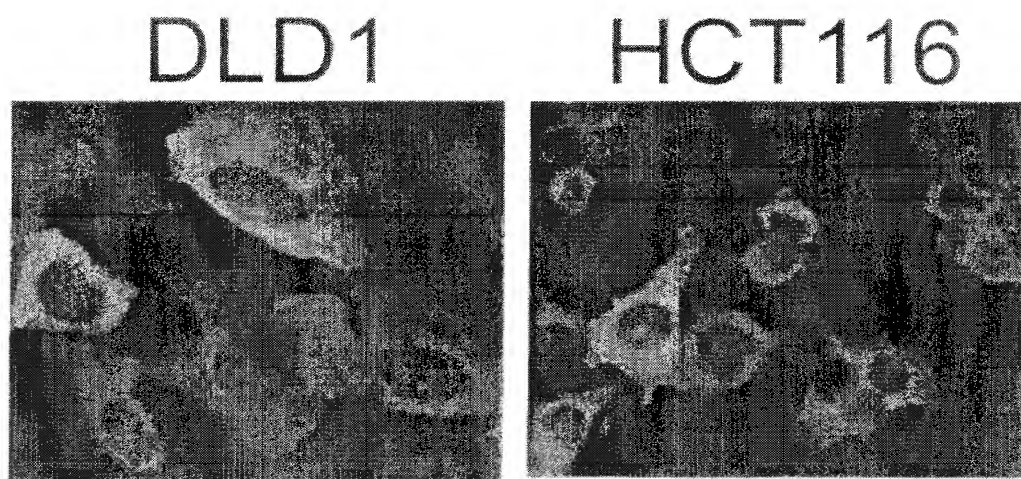


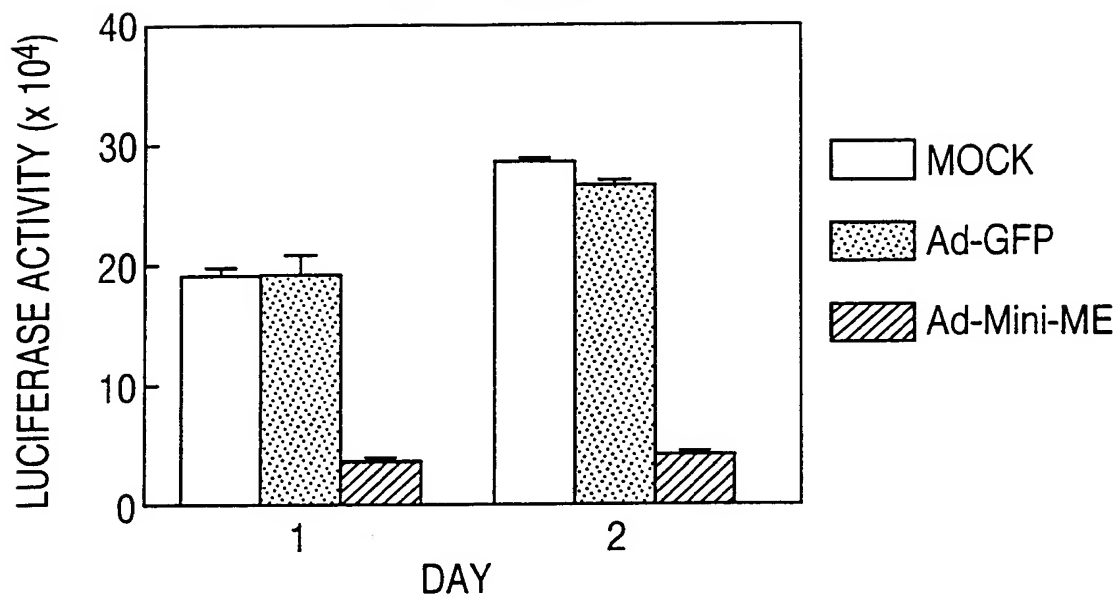
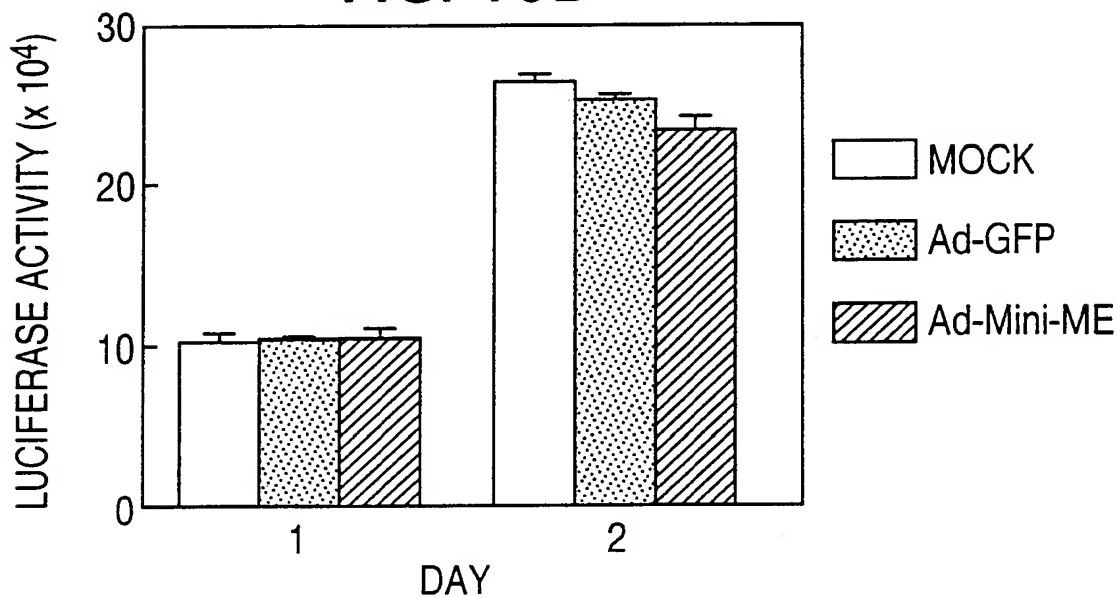
FIG. 9A



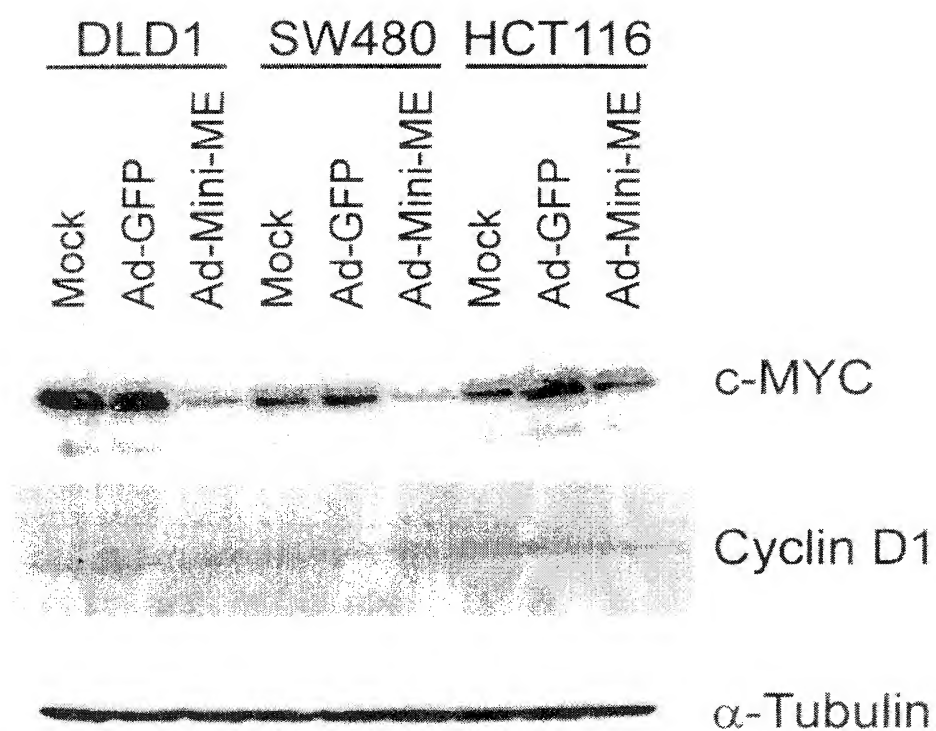
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FIG. 9B**FIG. 9C**

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FIG. 10A**FIG. 10B**

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FIG. II

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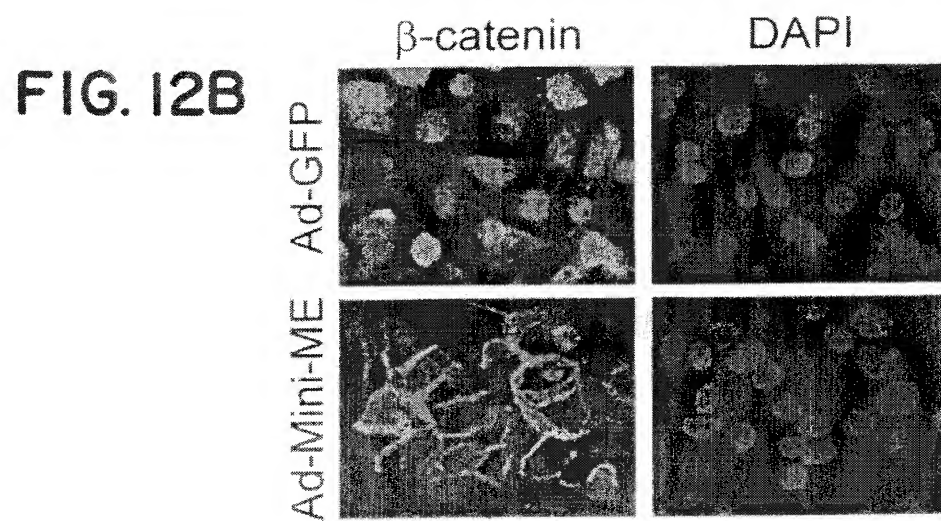
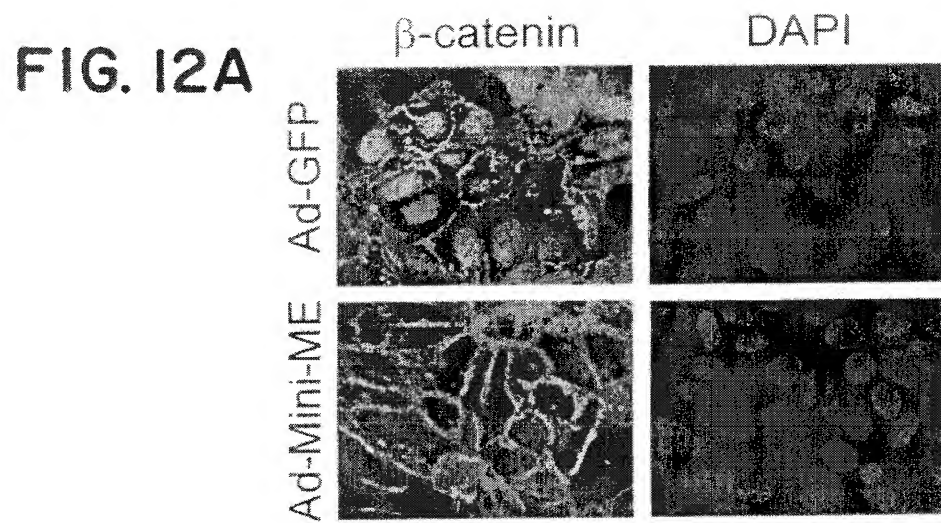


FIG. 13A**DLD1**

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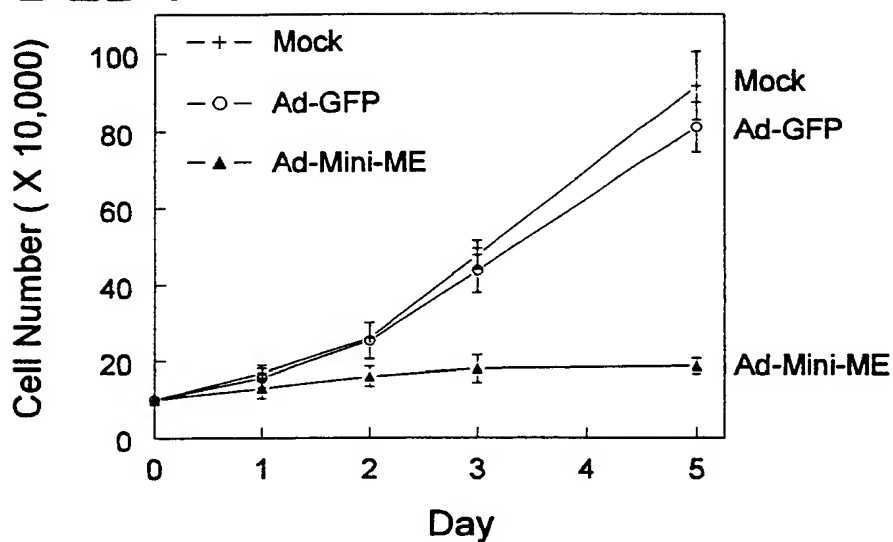
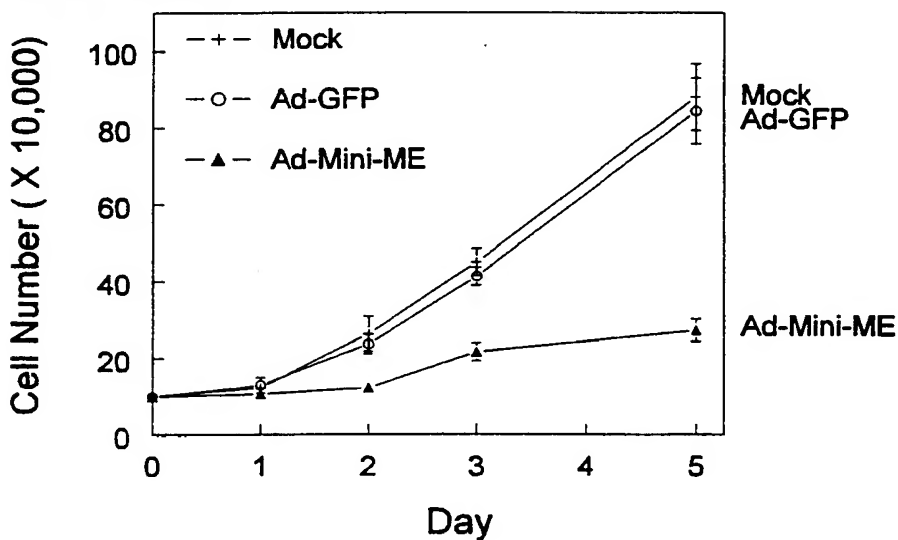
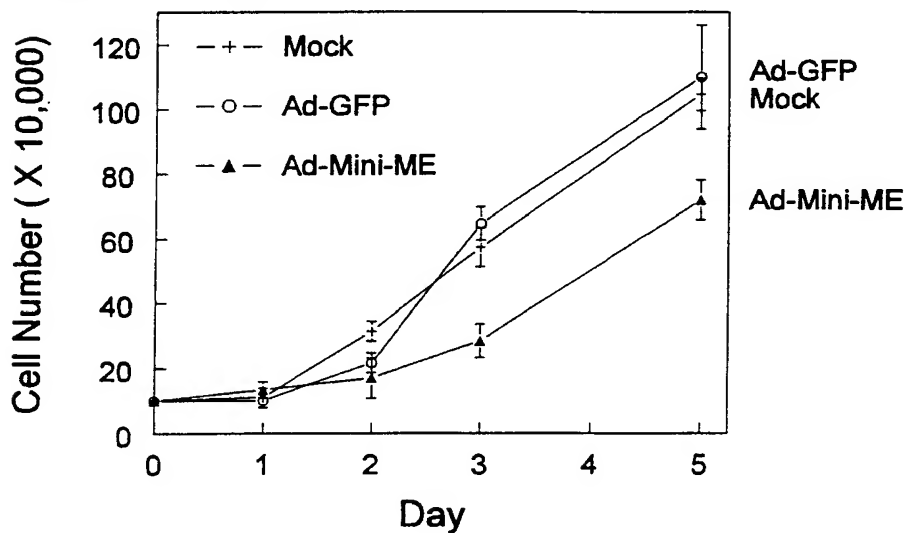
**SW480****HCT116**

FIG. 13B

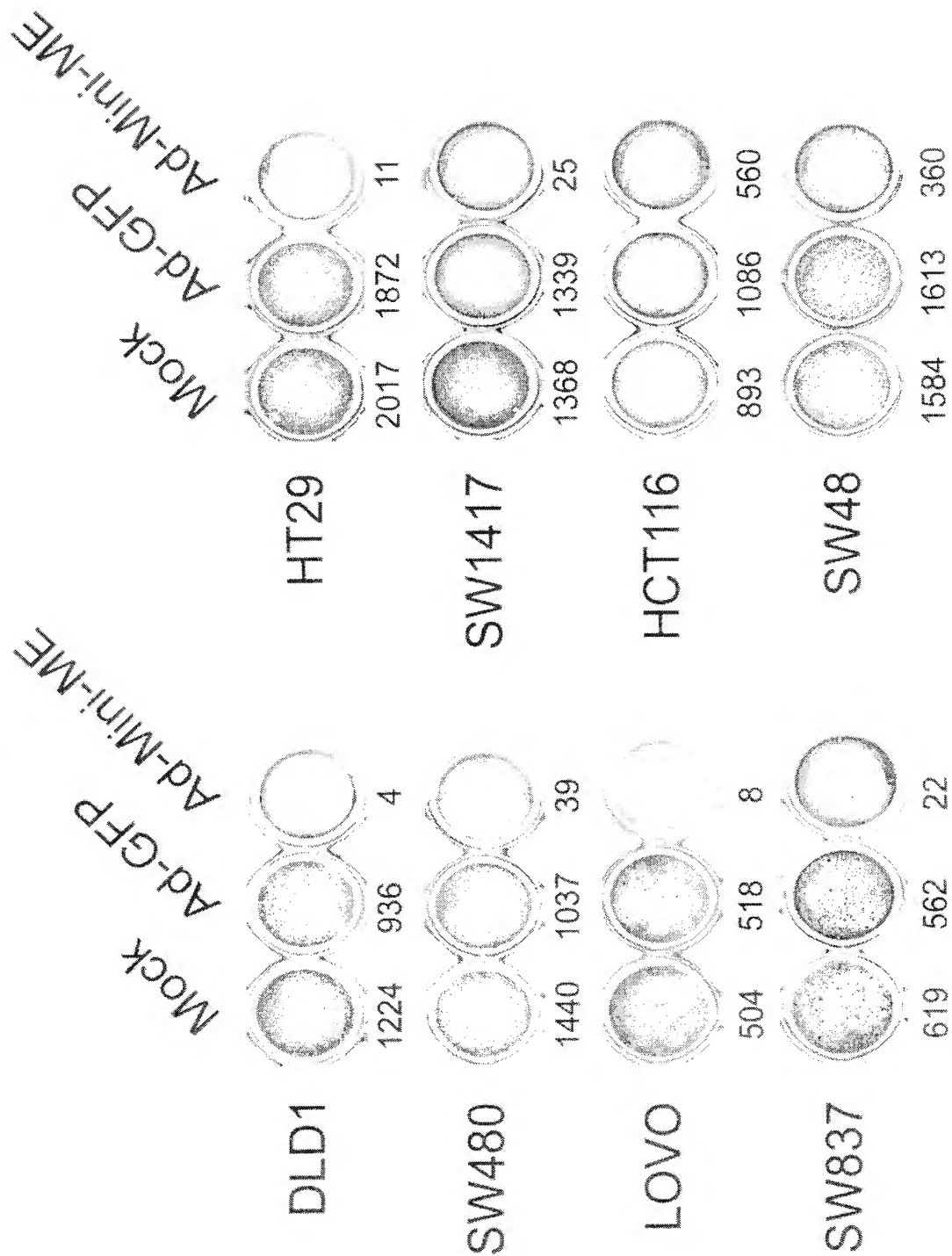


FIG. 14

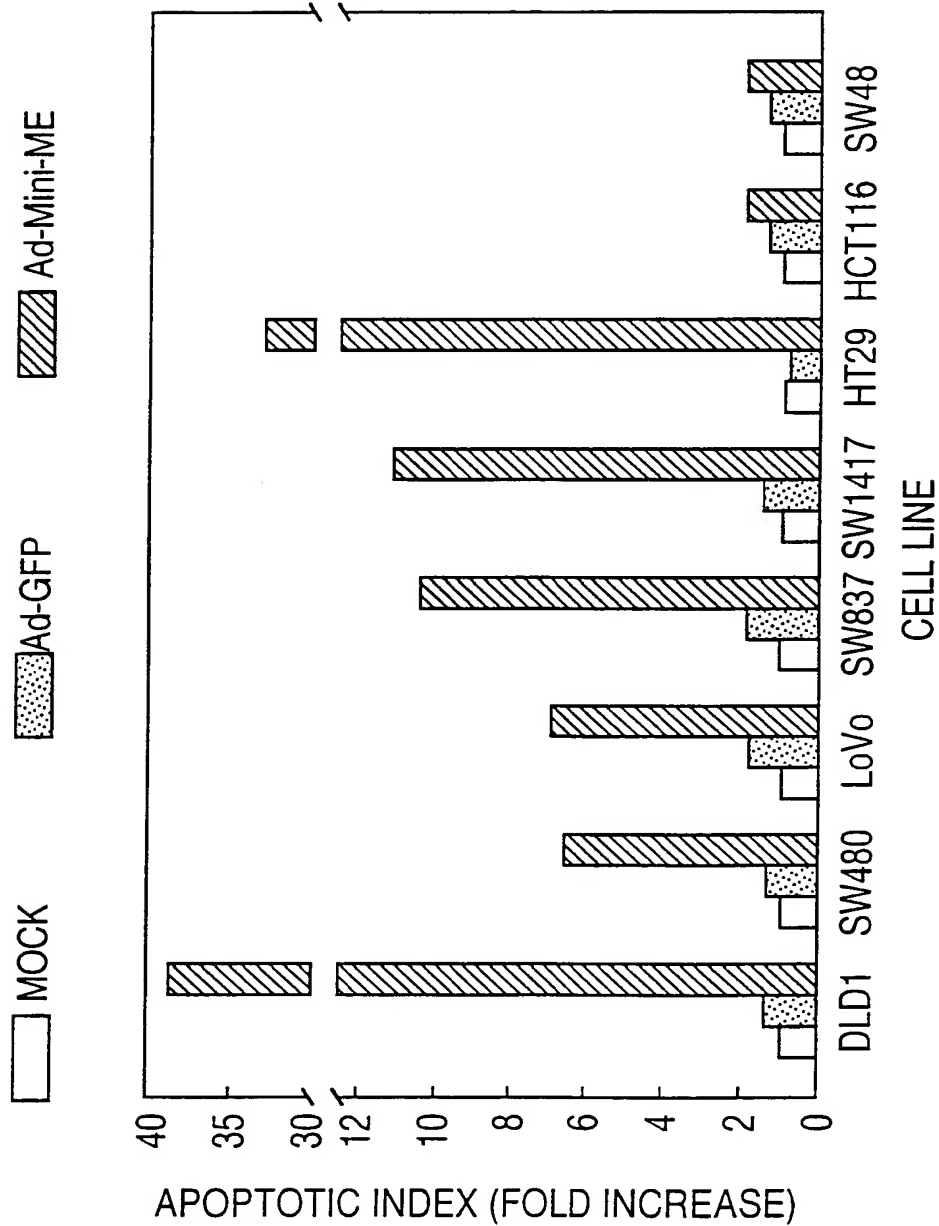
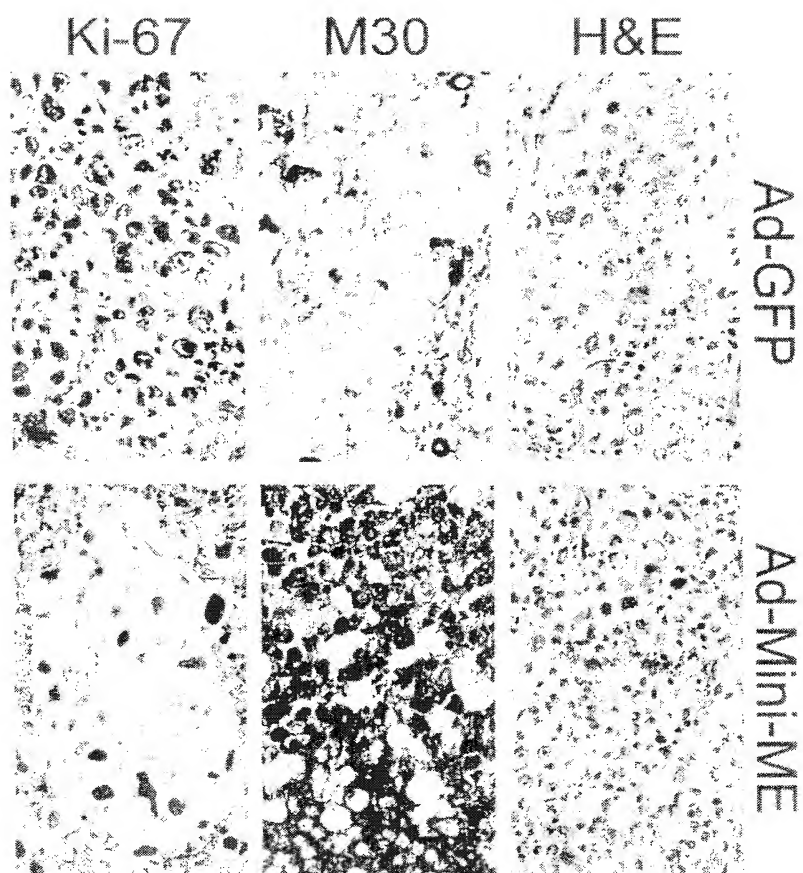


FIG. 15



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<170> FastSEQ for Windows Version 3.0

<210> 1
 <211> 2040
 <212> DNA
 <213> Homo sapiens

<400> 1

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<210> 2

<211> 2444

<212> DNA

<213> Homo sapiens

<400> 2

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gatttttttt	ggcttttctt	cctccttcat	ttttcttcca	aaattgctgc	tggtgggtga	300
aaaaaaaaatg	agcctccaga	gcgaacagga	ggagaagagc	tccgaaaact	cctcggcaga	360
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gctgtttaaa	gtctttgtag	cgtttaaaaa	atatatatat	atacataact	gttatgtagt	2400
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<210> 3
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 <212> DNA
 <213> Homo sapiens

<400> 3
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<210> 4
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 <212> DNA
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<400> 4
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<210> 5
 <211> 442
 <212> PRT
 <213> Homo sapiens

<400> 5
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 Glu Asn Ser Ser Ala Glu Arg Asp Leu Ala Asp Val Lys Ser Ser Leu
 35 40 45
 Val Asn Glu Ser Glu Thr Asn Gln Asn Ser Ser Ser Asp Ser Glu Ala
 50 55 60
 Glu Arg Arg Pro Pro Pro Arg Ser Glu Ser Phe Arg Asp Lys Ser Arg
 65 70 75 80
 Glu Ser Leu Glu Glu Ala Ala Lys Arg Gln Asp Gly Gly Leu Phe Lys
 85 90 95
 Gly Pro Pro Tyr Pro Gly Tyr Pro Phe Ile Met Ile Pro Asp Leu Thr
 100 105 110
 Ser Pro Tyr Leu Pro Lys Arg Ser Val Ser Pro Thr Ala Arg Thr Tyr
 115 120 125
 Leu Gln Met Lys Trp Pro Leu Leu Asp Val Gln Ala Gly Ser Leu Gln
 130 135 140
 Ser Arg Gln Ala Leu Lys Asp Ala Arg Ser Pro Ser Pro Ala His Ile
 145 150 155 160
 Val Ser Asn Lys Val Pro Val Val Gln His Pro His His Val His Pro
 165 170 175
 Leu Thr Pro Leu Ile Thr Tyr Ser Asn Glu His Phe Thr Pro Gly Asn
 180 185 190
 Pro Pro Pro His Leu Pro Ala Asp Val Asp Pro Lys Thr Gly Ile Pro
 195 200 205
 Arg Pro Pro His Pro Pro Asp Ile Ser Pro Tyr Tyr Pro Leu Ser Pro
 210 215 220
 Gly Thr Val Gly Gln Ile Pro His Pro Leu Gly Trp Leu Val Pro Gln
 225 230 235 240
 Gln Gly Gln Pro Val Tyr Pro Ile Thr Thr Gly Gly Phe Arg His Pro
 245 250 255
 Tyr Pro Thr Ala Leu Thr Val Asn Ala Ser Val Ser Arg Phe Pro Pro
 260 265 270
 His Met Val Pro Pro His His Thr Leu His Thr Thr Gly Ile Pro His
 275 280 285
 Pro Ala Ile Val Thr Pro Thr Val Lys Gln Glu Ser Ser Gln Ser Asp

290		295		300
Val Gly Ser Leu His Ser Ser Lys His Gln Asp Ser Lys Lys Glu Glu				
305		310		315
Glu Lys Lys Lys Pro His Ile Lys Lys Pro Leu Asn Ala Phe Met Leu				320
	325		330	335
Tyr Met Lys Glu Met Arg Ala Lys Val Val Ala Glu Cys Thr Leu Lys				
	340		345	350
Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg Trp His Ala Leu				
	355		360	365
Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala Arg Lys Glu Arg				
	370		375	380
Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala Arg Asp Asn Tyr				
385		390		395
Gly Lys Lys Lys Lys Arg Lys Arg Asp Lys Gln Pro Gly Glu Thr Asn				400
	405		410	415
Gly Glu Lys Lys Ser Ala Phe Ala Thr Tyr Lys Val Lys Ala Ala Ala				
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Ser Ala His Pro Leu Gln Met Glu Ala Tyr				
	435		440	

<210> 6
 <211> 596
 <212> PRT
 <213> Homo sapiens

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Glu Asn Ser Ser Ala Glu Arg Asp Leu Ala Asp Val Lys Ser Ser Leu	
	35
Val Asn Glu Ser Glu Thr Asn Gln Asn Ser Ser Ser Asp Ser Glu Ala	
	50
Glu Arg Arg Pro Pro Pro Arg Ser Glu Ser Phe Arg Asp Lys Ser Arg	
65	70
Glu Ser Leu Glu Glu Ala Ala Lys Arg Gln Asp Gly Gly Leu Phe Lys	
	85
Gly Pro Pro Tyr Pro Gly Tyr Pro Phe Ile Met Ile Pro Asp Leu Thr	
	100
Ser Pro Tyr Leu Pro Asn Gly Ser Val Ser Pro Thr Ala Arg Thr Tyr	
	115
Leu Gln Met Lys Trp Pro Leu Leu Asp Val Gln Ala Gly Ser Leu Gln	
	130
Ser Arg Gln Ala Leu Lys Asp Ala Arg Ser Pro Ser Pro Ala His Ile	
145	150
Val Ser Asn Lys Val Pro Val Val Gln His Pro His His Val His Pro	
	165
Leu Thr Pro Leu Ile Thr Tyr Ser Asn Glu His Phe Thr Pro Gly Asn	
	180
Pro Pro Pro His Leu Pro Ala Asp Val Asp Pro Lys Thr Gly Ile Pro	
	195
Arg Pro Pro His Pro Pro Asp Ile Ser Pro Tyr Tyr Pro Leu Ser Pro	
	210
Gly Thr Val Gly Gln Ile Pro His Pro Leu Gly Trp Leu Val Pro Gln	
225	230
Gln Gly Gln Pro Val Tyr Pro Ile Thr Thr Gly Gly Phe Arg His Pro	
	245
Tyr Pro Thr Ala Leu Thr Val Asn Ala Ser Val Ser Arg Phe Pro Pro	
	250

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                260                265                270
His Met Val Pro Pro His His Thr Leu His Thr Thr Gly Ile Pro His
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Pro Ala Ile Val Thr Pro Thr Val Lys Gln Glu Ser Ser Gln Ser Asp
                290                295                300
Val Gly Ser Leu His Ser Ser Lys His Gln Asp Ser Lys Lys Glu Glu
305                310                315                320
Glu Lys Lys Lys Pro His Ile Lys Lys Pro Leu Asn Ala Phe Met Leu
                325                330                335
Tyr Met Lys Glu Met Arg Ala Lys Val Val Ala Glu Cys Thr Leu Lys
                340                345                350
Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg Trp His Ala Leu
                355                360                365
Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala Arg Lys Glu Arg
                370                375                380
Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala Arg Asp Asn Tyr
385                390                395                400
Gly Lys Lys Lys Lys Arg Lys Arg Asp Lys Gln Pro Gly Glu Thr Asn
                405                410                415
Glu His Ser Glu Cys Phe Leu Asn Pro Cys Leu Ser Leu Pro Pro Ile
                420                425                430
Thr Asp Leu Ser Ala Pro Lys Lys Cys Arg Ala Arg Phe Gly Leu Asp
                435                440                445
Gln Gln Asn Asn Trp Cys Gly Pro Cys Arg Arg Lys Lys Lys Cys Val
450                455                460
Arg Tyr Ile Gln Gly Glu Gly Ser Cys Leu Ser Pro Pro Ser Ser Asp
465                470                475                480
Gly Ser Leu Leu Asp Ser Pro Pro Pro Ser Pro Asn Leu Leu Gly Ser
                485                490                495
Pro Pro Arg Asp Ala Lys Ser Gln Thr Glu Gln Thr Gln Pro Leu Ser
                500                505                510
Leu Ser Leu Lys Pro Asp Pro Leu Ala His Leu Ser Met Met Pro Pro
                515                520                525
Pro Pro Ala Leu Leu Leu Ala Glu Ala Thr His Lys Ala Ser Ala Leu
                530                535                540
Cys Pro Asn Gly Ala Leu Asp Leu Pro Pro Ala Ala Leu Gln Pro Ala
545                550                555                560
Ala Pro Ser Ser Ser Ile Ala Gln Pro Ser Thr Ser Trp Leu His Ser
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His Ser Ser Leu Ala Gly Thr Gln Pro Gln Pro Leu Ser Leu Val Thr
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Lys Ser Leu Glu
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<210> 7

<211> 2973

<212> PRT

<213> Homo sapiens

<400> 7

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Lys Met Glu Asn Ser Asn Leu Arg Gln Glu Leu Glu Asp Asn Ser Asn
                20                25                30
His Leu Thr Lys Leu Glu Thr Glu Ala Ser Asn Met Lys Glu Val Leu
                35                40                45
Lys Gln Leu Gln Gly Ser Ile Glu Asp Glu Ala Met Ala Ser Ser Gly
50                55                60
Gln Ile Asp Leu Leu Glu Arg Leu Lys Glu Leu Asn Leu Asp Ser Ser

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65					70					75					80
Asn	Phe	Pro	Gly	Val	Lys	Leu	Arg	Ser	Lys	Met	Ser	Leu	Arg	Ser	Tyr
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Gly	Ser	Arg	Glu	Gly	Ser	Val	Ser	Ser	Arg	Ser	Gly	Glu	Cys	Ser	Pro
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Val	Pro	Met	Gly	Ser	Phe	Pro	Arg	Arg	Gly	Phe	Val	Asn	Gly	Ser	Arg
		115					120					125			
Glu	Ser	Thr	Gly	Tyr	Leu	Glu	Glu	Leu	Glu	Lys	Glu	Arg	Ser	Leu	Leu
		130				135					140				
Leu	Ala	Asp	Leu	Asp	Lys	Glu	Glu	Lys	Glu	Lys	Asp	Trp	Tyr	Tyr	Ala
145					150					155					160
Gln	Leu	Gln	Asn	Leu	Thr	Lys	Arg	Ile	Asp	Ser	Leu	Pro	Leu	Thr	Glu
			165						170					175	
Asn	Phe	Ser	Leu	Gln	Thr	Asp	Met	Thr	Arg	Arg	Gln	Leu	Glu	Tyr	Glu
			180					185					190		
Ala	Arg	Gln	Ile	Arg	Val	Ala	Met	Glu	Glu	Gln	Leu	Gly	Thr	Cys	Gln
		195					200					205			
Asp	Met	Glu	Lys	Arg	Ala	Gln	Arg	Arg	Ile	Ala	Arg	Ile	Gln	Gln	Ile
		210				215					220				
Glu	Lys	Asp	Ile	Leu	Arg	Ile	Arg	Gln	Leu	Leu	Gln	Ser	Gln	Ala	Thr
225					230					235					240
Glu	Ala	Glu	Arg	Ser	Gln	Asn	Lys	His	Glu	Thr	Gly	Ser	His	Asp	
			245					250					255		
Ala	Glu	Arg	Gln	Asn	Glu	Gly	Gln	Gly	Val	Gly	Glu	Ile	Asn	Met	Ala
			260					265					270		
Thr	Ser	Gly	Asn	Gly	Gln	Gly	Ser	Thr	Thr	Arg	Met	Asp	His	Glu	Thr
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Ala	Ser	Val	Leu	Ser	Ser	Ser	Ser	Thr	His	Ser	Ala	Pro	Arg	Arg	Leu
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Thr	Ser	His	Leu	Gly	Thr	Lys	Val	Glu	Met	Val	Tyr	Ser	Leu	Leu	Ser
305					310					315					320
Met	Leu	Gly	Thr	His	Asp	Lys	Asp	Asp	Met	Ser	Arg	Thr	Leu	Leu	Ala
			325						330					335	
Met	Ser	Ser	Ser	Gln	Asp	Ser	Cys	Ile	Ser	Met	Arg	Gln	Ser	Gly	Cys
			340					345					350		
Leu	Pro	Leu	Leu	Ile	Gln	Leu	Leu	His	Gly	Asn	Asp	Lys	Asp	Ser	Val
		355					360					365			
Leu	Leu	Gly	Asn	Ser	Arg	Gly	Ser	Lys	Glu	Ala	Arg	Ala	Arg	Ala	Ser
		370				375					380				
Ala	Ala	Leu	His	Asn	Ile	Ile	His	Ser	Gln	Pro	Asp	Asp	Lys	Arg	Gly
385					390					395					400
Arg	Arg	Glu	Ile	Arg	Val	Leu	His	Leu	Leu	Glu	Gln	Ile	Arg	Ala	Tyr
			405						410					415	
Cys	Glu	Thr	Cys	Trp	Glu	Trp	Gln	Glu	Ala	His	Glu	Pro	Gly	Met	Asp
			420					425					430		
Gln	Asp	Lys	Asn	Pro	Met	Pro	Ala	Pro	Val	Glu	His	Gln	Ile	Cys	Pro
		435					440					445			
Ala	Val	Cys	Val	Leu	Met	Lys	Leu	Ser	Phe	Asp	Glu	Glu	His	Arg	His
		450				455					460				
Ala	Met	Asn	Glu	Leu	Gly	Gly	Leu	Gln	Ala	Ile	Ala	Glu	Leu	Leu	Gln
465					470					475					480
Val	Asp	Cys	Glu	Met	Tyr	Gly	Leu	Thr	Asn	Asp	His	Tyr	Ser	Ile	Thr
			485						490					495	
Leu	Arg	Arg	Tyr	Ala	Gly	Met	Ala	Leu	Thr	Asn	Leu	Thr	Phe	Gly	Asp
			500					505					510		
Val	Ala	Asn	Lys	Ala	Thr	Leu	Cys	Ser	Met	Lys	Gly	Cys	Met	Arg	Ala
		515					520					525			
Leu	Val	Ala	Gln	Leu	Lys	Ser	Glu	Ser	Glu	Asp	Leu	Gln	Gln	Val	Ile
		530				535						540			

Ala Ser Val Leu Arg Asn Leu Ser Trp Arg Ala Asp Val Asn Ser Lys
 545 550 555 560
 Lys Thr Leu Arg Glu Val Gly Ser Val Lys Ala Leu Met Glu Cys Ala
 565 570 575
 Leu Glu Val Lys Lys Glu Ser Thr Leu Lys Ser Val Leu Ser Ala Leu
 580 585 590
 Trp Asn Leu Ser Ala His Cys Thr Glu Asn Lys Ala Asp Ile Cys Ala
 595 600 605
 Val Asp Gly Ala Leu Ala Phe Leu Val Gly Thr Leu Thr Tyr Arg Ser
 610 615 620
 Gln Thr Asn Thr Leu Ala Ile Ile Glu Ser Gly Gly Gly Ile Leu Arg
 625 630 635 640
 Asn Val Ser Ser Leu Ile Ala Thr Asn Glu Asp His Arg Gln Ile Leu
 645 650 655
 Arg Glu Asn Asn Cys Leu Gln Thr Leu Leu Gln His Leu Lys Ser His
 660 665 670
 Ser Leu Thr Ile Val Ser Asn Ala Cys Gly Thr Leu Trp Asn Leu Ser
 675 680 685
 Ala Arg Asn Pro Lys Asp Gln Glu Ala Leu Trp Asp Met Gly Ala Val
 690 695 700
 Ser Met Leu Lys Asn Leu Ile His Ser Lys His Lys Met Ile Ala Met
 705 710 715 720
 Gly Ser Ala Ala Ala Leu Arg Asn Leu Met Ala Asn Arg Pro Ala Lys
 725 730 735
 Tyr Lys Asp Ala Asn Ile Met Ser Pro Gly Ser Ser Leu Pro Ser Leu
 740 745 750
 His Val Arg Lys Gln Lys Ala Leu Glu Ala Glu Leu Asp Ala Gln His
 755 760 765
 Leu Ser Glu Thr Phe Asp Asn Ile Asp Asn Leu Ser Pro Lys Ala Ser
 770 775 780
 His Arg Ser Lys Gln Arg His Lys Gln Ser Leu Tyr Gly Asp Tyr Val
 785 790 795 800
 Phe Asp Thr Asn Arg His Asp Asp Asn Arg Ser Asp Asn Phe Asn Thr
 805 810 815
 Gly Asn Met Thr Val Leu Ser Pro Tyr Leu Asn Thr Thr Val Leu Pro
 820 825 830
 Ser Ser Ser Ser Ser Arg Gly Ser Leu Asp Ser Ser Arg Ser Glu Lys
 835 840 845
 Asp Arg Ser Leu Glu Arg Glu Arg Gly Ile Gly Leu Gly Asn Tyr His
 850 855 860
 Pro Ala Thr Glu Asn Pro Gly Thr Ser Ser Lys Arg Gly Leu Gln Ile
 865 870 875 880
 Ser Thr Thr Ala Ala Gln Ile Ala Lys Val Met Glu Glu Val Ser Ala
 885 890 895
 Ile His Thr Ser Gln Glu Asp Arg Ser Ser Gly Ser Thr Thr Glu Leu
 900 905 910
 His Cys Val Thr Asp Glu Arg Asn Ala Leu Arg Arg Ser Ser Ala Ala
 915 920 925
 His Thr His Ser Asn Thr Tyr Asn Phe Thr Lys Ser Glu Asn Ser Asn
 930 935 940
 Arg Thr Cys Ser Met Pro Tyr Ala Lys Leu Glu Tyr Lys Arg Ser Ser
 945 950 955 960
 Asn Asp Ser Leu Asn Ser Val Ser Ser Ser Asp Gly Tyr Gly Lys Arg
 965 970 975
 Gly Gln Met Lys Pro Ser Ile Glu Ser Tyr Ser Glu Asp Asp Glu Ser
 980 985 990
 Lys Phe Cys Ser Tyr Gly Gln Tyr Pro Ala Asp Leu Ala His Lys Ile
 995 1000 1005
 His Ser Ala Asn His Met Asp Asp Asn Asp Gly Glu Leu Asp Thr Pro

1010	1015	1020
Ile Asn Tyr Ser Leu Lys Tyr Ser Asp Glu Gln Leu Asn Ser Gly Arg		
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Gln Ser Pro Ser Gln Asn Glu Arg Trp Ala Arg Pro Lys His Ile Ile		1040
	1045	1050
Glu Asp Glu Ile Lys Gln Ser Glu Gln Arg Gln Ser Arg Asn Gln Ser		1055
	1060	1065
Thr Thr Tyr Pro Val Tyr Thr Glu Ser Thr Asp Asp Lys His Leu Lys		1070
	1075	1080
Phe Gln Pro His Phe Gly Gln Gln Glu Cys Val Ser Pro Tyr Arg Ser		1085
	1090	1095
Arg Gly Ala Asn Gly Ser Glu Thr Asn Arg Val Gly Ser Asn His Gly		1100
1105	1110	1115
Ile Asn Gln Asn Val Ser Gln Ser Leu Cys Gln Glu Asp Asp Tyr Glu		1120
	1125	1130
Asp Asp Lys Pro Thr Asn Tyr Ser Glu Arg Tyr Ser Glu Glu Gln		1135
	1140	1145
His Glu Glu Glu Glu Arg Pro Thr Asn Tyr Ser Ile Lys Tyr Asn Glu		1150
	1155	1160
Glu Lys Arg His Val Asp Gln Pro Ile Asp Tyr Ser Leu Lys Tyr Ala		1165
	1170	1175
Thr Asp Ile Pro Ser Ser Gln Lys Gln Ser Phe Ser Phe Ser Lys Ser		1180
1185	1190	1195
Ser Ser Gly Gln Ser Ser Lys Thr Glu His Met Ser Ser Ser Ser Glu		1200
	1205	1210
Asn Thr Ser Thr Pro Ser Ser Asn Ala Lys Arg Gln Asn Gln Leu His		1215
	1220	1225
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8 March 2001 (08.03.2001)

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(72) Inventors; and

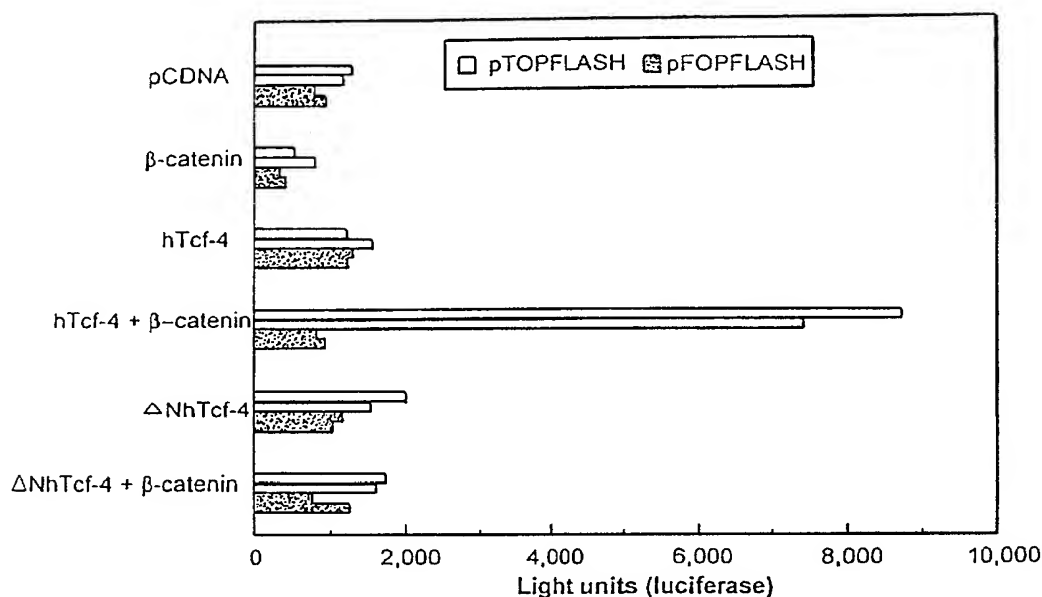
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(US). **HE, Tong-Chuan** [US/US]; 6019 S. Ingleside
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20001-4597 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,

[Continued on next page]

(54) Title: BETA-CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER



(57) Abstract: A recombinant adenovirus (Ad-Mini-ME) which constitutively expresses the central third of APC includes all of the known β-catenin binding repeats. When expressed in colon cancer cells, Ad-Mini-ME blocked the nuclear translocation of β-catenin and inhibited β-catenin/Tcf-4-mediated transactivation. Accordingly, expression of endogenous targets of the APC/β-catenin/Tcf-4 pathway were down-regulated. Ad-Mini-ME infection of colorectal cancer cell lines with mutant APC but wild-type β-catenin resulted in substantial growth arrest followed by apoptosis. These findings suggest that the β-catenin binding domain in the central third of APC is sufficient for its tumor suppression activity.

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LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

- (84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 00/23635

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/47 C12N15/12 C12N15/62 A61P35/00 A61K38/17
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, SCISEARCH, MEDLINE, CHEM ABS Data, EMBASE, BIOTECHNOLOGY ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORIN P J ET AL: "Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 275, 21 March 1997 (1997-03-21), pages 1787-1790, XP002088507 ISSN: 0036-8075	6,8,23, 24
Y	the whole document	1,3,4, 10-19, 21,22, 25,26
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- *Z* document member of the same patent family

Date of the actual completion of the international search

22 February 2001

Date of mailing of the international search report

19/03/2001

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Fax: (+31-70) 340-3016

Authorized officer

ALCONADA RODRIG., A

INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 00/23635

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUBINFELD BONNEE ET AL: "Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene." CANCER RESEARCH, vol. 57, no. 20, 1997, pages 4624-4630, XP000979563 ISSN: 0008-5472	1,4,6,8, 21-24
Y	page 4625, right-hand column, last paragraph -page 4626, left-hand column, paragraph 1; figures 1,5 ---	10-12, 18,25,26
X	US 5 910 418 A (KINZLER KENNETH W ET AL) 8 June 1999 (1999-06-08) column 4, line 64 -column 5, line 8; example 1	1,4,6,8, 21-24
Y	column 4, line 53-63 ---	3
X	JOHNSON S ET AL: "CASPASE-MEDIATED CLEAVAGE OF APC RESULTS IN AN AMINO-TERMINAL FRAGMENT WITH AN INTACT ARMADILLO REPEAT DOMAIN" FASEB JOURNAL, FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, US, vol. 13, February 1999 (1999-02), pages 339-346, XP000917975 ISSN: 0892-6638 page 342, left-hand column, last paragraph; figure 2 ---	6,8,23, 24
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